

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/00, A61K 37/02 C12P 21/08, C12N 15/12, 5/10 C12Q 1/68, A61K 39/395		A1	(11) International Publication Number: WO 92/17497 (43) International Publication Date: 15 October 1992 (15.10.92)
(21) International Application Number: PCT/US92/02317 (22) International Filing Date: 23 March 1992 (23.03.92) (30) Priority data: 677,211 29 March 1991 (29.03.91) US 810,782 19 December 1991 (19.12.91) US (71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US). (72) Inventors: LEE, James ; 705 Shelter Creek Lane, San Bruno, CA 94066 (US). HOLMES, William, E. ; 29 Eastlake Avenue, Pacifica, CA 94044 (US). WOOD, William, I. ; 1400 Terrytown Street, San Mateo, CA 94402 (US).		(74) Agents: HENSLEY, Max, D. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: HUMAN PF4A RECEPTORS AND THEIR USE (57) Abstract cDNAs encoding a class of novel PF4ARs has been identified in human tissue. Provided herein are nucleic acid sequences of three PF4ARs (including the human IL-8 receptor) useful as a diagnostic and in the recombinant preparation of PF4ARs. The PF4ARs are used in the preparation and purification of antibodies capable of binding to the receptors, and in diagnostic assays.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT:

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Togo
DE	Germany	MC	Monaco	TC	Togo
DK	Denmark			US	United States of America

HUMAN PF4A RECEPTORS AND THEIR USE

This invention relates to the field of assaying platelet factor 4 superfamily members (hereafter "PF4A") and the preparation of agonists and antagonists to the members of this family.

5

BACKGROUND OF THE INVENTION

While interleukin-8 was initially identified as a chemoattractant for neutrophils, and was known to bind a receptor on neutrophils⁸⁻¹⁰, it has in addition a wide range of pro-inflammatory activities including the stimulation of degranulation and the upregulation of the cell adhesion molecule MAC-1 and of the complement receptor CR1¹. IL-8 can also
10 mediate the inhibition of the adherence of neutrophils to activated endothelial cells².

IL-8 is a member of a family of ten or more pro-inflammatory cytokines with an M_r ~ 10,000¹. This larger family of proteins is called the platelet factor 4 superfamily (Wolpe *et al.*, FASEB J. 3:2565-73 [1989]). Some members of the platelet factor 4 superfamily, in general the subset referred to as CXC peptides (including IL-8), possess neutrophil agonist
15 activity, e.g. NAP-2, MIP-2, platelet factor 4 and NAP-3 (MGSA/gro). Other members of this family, the C-C peptides, are not neutrophil agonists. Hereafter "PF4A" means the PF4 superfamily.

It is an object of this invention to identify receptors for the PF4A superfamily (hereinafter "PF4AR").

20

It is another object of this invention to obtain DNA encoding or hybridizing to these receptors, and to express the receptors in host cells.

It is an additional object of this invention to provide isolates of PF4AR for diagnostic and therapeutic purposes.

A still further object is to obtain DNA encoding variants of such receptors and to
25 prepare such variants in recombinant cell culture.

These and other objects of this invention will be apparent from the specification as a whole.

SUMMARY OF THE INVENTION

These objects are accomplished, in one aspect, by providing an isolated novel PF4AR
30 polypeptides, including polypeptides that are related structurally to the PF4AR. Members of this class of polypeptide are hereafter generically termed PF4AR, and include derivatives and variants thereof.

In another aspect, the invention provides a composition comprising the PF4AR that is free of contaminating polypeptides of the animal species from which the PF4AR is derived.

35

The PF4AR or fragments thereof (which also may be synthesized by chemical methods) are fused (by recombinant expression or *in vitro* covalent methods) to an immunogenic polypeptide and this fusion polypeptide, in turn, is used to immunize an animal to raise antibodies against a PF4AR epitope. Anti-PF4AR antibodies are recovered from the serum

-2-

of immunized animals. Alternatively, monoclonal antibodies are prepared from cells of the immunized animal in conventional fashion.

Anti-PF4AR antibodies are useful particularly in the diagnosis (*in vitro* or *in vivo*) or (when immobilized on an insoluble matrix) the purification of the PF4AR.

5 Substitutional, deletional, or insertional variants of the PF4AR are prepared by *in vitro* or recombinant methods and screened for immuno-crossreactivity with the PF4AR and for PF4AR antagonist or agonist activity.

10 The PF4AR also is derivatized *in vitro* to prepare immobilized PF4AR and labeled PF4AR, particularly for purposes of diagnosis of PF4AR or its antibodies, or for affinity purification of PF4AR antibodies.

The PF4AR, its derivatives, or its antibodies are formulated into physiologically acceptable vehicles, especially for therapeutic use. Such vehicles include sustained-release formulations of the PF4AR.

15 In still other aspects, the invention provides an isolated nucleic acid molecule encoding the PF4AR, labeled or unlabeled, and a nucleic acid sequence that is complementary to, or hybridizes under suitable conditions to a nucleic acid sequence encoding the PF4AR.

20 In addition, the invention provides a replicable vector comprising the nucleic acid molecule encoding the PF4AR operably linked to control sequences recognized by a host transformed by the vector; host cells transformed with the vector; and a method of using a nucleic acid molecule encoding the PF4AR to effect the production of PF4AR, comprising expressing the nucleic acid molecule in a culture of the transformed host cells and recovering the PF4AR from the host cell culture. The nucleic acid sequence is also useful in hybridization assays for PF4AR nucleic acid. The recombinant host cells are particularly useful in assaying the appropriate PF4A members.

25 In further embodiments, the invention provides a method for producing PF4AR comprising inserting into the DNA of a cell containing the nucleic acid encoding the PF4AR a transcription modulatory element in sufficient proximity and orientation to the PF4AR nucleic acid to influence or destroy transcription of DNA encoding a biologically active PF4AR, with an optional further step comprising culturing the cell containing the transcription
30 modulatory element and the PF4AR nucleic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the high affinity binding of IL-8 to COS cells transfected with clone PRK5B.il8r1.1. a, Competition with unlabelled IL-8 or fMLP. b, Scatchard analysis of the IL-8 competition data; apparent K_d = 3.6 nM, average of 820,000 binding sites/cell. Similar
35 competitions with human neutrophils gave K_d = 1.1 nM, 31000 binding sites/cell.

Figs. 2a-2c (SEQ ID NO.1) (hereinafter referred to collectively as Fig. 2) depicts the amino acid and nucleotide sequences of the IL-8 receptor cDNA insert from clone PRK5B.il8r1.1. The seven putative transmembrane domains are shown. There are 4

-3-

extracellular segments and 4 intracellular segments, each being separated by one of the transmembrane domains. The extracellular segments are approximately delineated by residues 1-39, 99-111, 134-154, 175-203 and 265-290. The IL-8 receptor contains 3 potential N-linked glycosylation sites in the first extracellular region and 3 more in the third extracellular loop.

Fig. 3a depicts the flow cytometry determination of the intracellular Ca^{++} response of transfected human IL-8 and fMLP receptors to their ligands. Human embryonic kidney 293 cells were transfected by electroporation¹⁹ with IL-8 receptor (clone pRK5B.il8r1.1), fMLP receptor (human fMLP receptor cDNA³ in the vector pRK5), or vector (pRK5B¹⁸) DNA. After two days, the cells were loaded with 2 μ M indo-1 acetoxymethyl ester in RPMI medium for 30 min at 37°. Intracellular Ca^{++} was measured with a Coulter 753 flow cytometer using the ratio of 405 and 525 nm fluorescence²³.

Fig. 3b illustrates the percent of cells above 400 nM Ca^{++} for the time period after addition of IL-8 (about 15 sec. into each run).

Figs. 4 (SEQ ID NO.2) and 5 (SEQ ID NO.3) depict the DNA and an imputed polypeptide sequences for two additional PF4AR members identified by probing lambda libraries from a human monocyte-like cell line (HL-60) and human PBLs using a large fragment of the IL-8 receptor DNA.

Detailed Description of the Invention

We have isolated by expression cloning a cDNA encoding the human neutrophil IL-8 receptor together with two other homologous receptors. The amino acid sequence shows that the IL-8 receptor is a member of the G-protein coupled receptor family with clear similarity (29 % amino acid identity) to the human neutrophil receptors for the chemoattractants f-Met-Leu-Phe³ and C5a⁴. Although the IL-8 receptor sequence may be the human homologue of what has been identified as the isoform of the rabbit f-Met-Leu-Phe receptor⁵, we show that when transfected into mammalian cells, this receptor clone confers high affinity binding to IL-8 and produces a transient Ca^{++} mobilization in response to IL-8 with no binding or response to f-Met-Leu-Phe.

A COS cell expression cloning strategy^{6,7} was used to isolate clones encoding the IL-8 receptor. A cDNA library constructed from human neutrophil mRNA in the mammalian expression vector pRK5B was transfected into COS-7 cells as pools of 2500 clones, and the cells screened for the binding of ¹²⁵I-IL-8. One positive pool from the first 58 transfections was partitioned into smaller pools until a pure clone (pRK5B.il8r1.1) was obtained. Figure 1 shows the competition of ¹²⁵I-IL-8 binding by unlabelled IL-8 to COS cells transfected with the isolated clone. Analysis of this data gives a K_d of 3.6 nM for IL-8 binding which is within the range of 0.8 to 4 nM reported for IL-8 binding to human neutrophils⁸⁻¹⁰. There is no competition of the IL-8 binding by the chemotactic peptide f-Met-Leu-Phe (fMLP).

-4-

The DNA sequence of the isolated cDNA clone (Fig. 2) contains a single long open reading frame beginning with a methionine residue that matches the consensus expected for a translation initiation site¹¹. This open reading frame encodes a protein of 350 amino acids (translated M; 39.5 kD). The amino acid sequence shares several features with the G-protein coupled receptors of the rhodopsin superfamily including seven hydrophobic domains that are presumed to span the cell membrane and N-linked glycosylation sites near the N-terminus¹² (see below).

The encoded amino acid sequence is the most similar to a recently cloned sequence for the rabbit fMLP receptor⁵. The similarity is sufficiently high (79% amino acid identity overall with multiple stretches of more than 20 contiguous amino acid matches) that these two sequences may well be species homologs of the same receptor. The human fMLP receptor has also been cloned³; it has only 26% amino acid identity with the rabbit fMLP receptor (and 29% identity to the human IL-8 receptor presented here). The considerable divergence between the rabbit and human fMLP receptor amino acid sequences has lead to the suggestion in the art (now believed to be possibly erroneous) that these may be two isoforms of the fMLP receptor⁵.

Neutrophils respond to the chemoattractants IL-8 and fMLP with a rapid, transient increase in the intracellular free Ca^{++} concentration^{1,13}. In order to verify the identification of the clone isolated here as the IL-8 receptor, we have determined the intracellular Ca^{++} response of transfected cells to added IL-8 as well as fMLP. We have used parallel experiments with transfected human fMLP receptor or with the expression vector as controls. Flow cytometer analysis shows a clear transient increase in intracellular Ca^{++} for the transfected IL-8 receptor in response to IL-8. No response is found to fMLP. Conversely, cells transfected with the human fMLP receptor respond to fMLP but not to IL-8. No response to either chemoattractant is found in vector transfected cells. Only a subset of the cells are expected to respond in these experiments as the transfection efficiency is estimated to be 15-25%. Binding experiments¹⁴ also failed to detect any binding of ³H-fMLP to the expressed IL-8 receptor or ¹²⁵I-IL-8 to the expressed human fMLP receptor. These experiments clearly demonstrate the specificity of the two receptors for their respective ligands; a result expected based on the lack of binding competition between IL-8 and fMLP for neutrophils¹. These results also demonstrate that the cloned receptors function in second message signaling in response to ligand binding.

Blot hybridization of the cloned IL-8 receptor cDNA to human neutrophil mRNA, shows strong bands of 2.4 and 3.0 kb as well as a fainter band at 3.5 kb. While it is clear from the DNA sequence data presented in Fig. 2 that the mRNA for the receptor has a long 3' untranslated region, additional work will be needed to establish whether the multiple RNA bands are due to multiple polyadenylation sites. No hybridization was detected to mRNA from U266 or Jurkat cell lines, which are of the B cell and T cell lineages. Northern hybridization

was found for mRNA from the monocyte cell line U937 as well, in spite of the reports of low levels of IL-8 binding to these cells^{9,10}.

Alignment of the receptor sequences for the three neutrophil chemoattractants IL-8, fMLP³, and C5a⁴ shows that they form a subfamily of the G-protein coupled receptors with 29-34 % amino acid identity. This subfamily has a short third intracellular loop as compared with other G-protein coupled receptors such as the β -adrenergic¹² or muscarinic acetylcholine receptors¹⁶. This loop contains determinants at least partially responsible for the binding of G-proteins to the receptors¹². The intracellular C-terminal region of the IL-8 receptor, while not very similar to that of the fMLP and C5a receptors does preserve a high number of serine and threonine residues that may function as phosphorylation sites. As has been noted for the C5a receptor⁴, the N-terminal extracellular region for the IL-8 receptor has several acidic residues. These may aid in the binding of IL-8 which is quite basic (pI ~ 9.5).

I. Definitions

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims:

The term "PF4AR" is defined as a polypeptide having a qualitative biological activity in common with the polypeptides of Figs. 2, 4, or 5. Optionally, PF4AR will have at least 30% and ordinarily 75% amino acid sequence identity with any of the polypeptides of Figs. 2, 4 or 5. Optionally, PF4AR excludes the rabbit fMLP receptor⁵, the human fMLP receptor², the human C5a receptor⁴, and/or the receptor described by Murphy *et al.*, Science 253:1280 (1991).

Identity or homology with respect to a PF4AR is defined herein to be the percentage of amino acid residues in the candidate sequence that are identical with the residues in Figs. 2, 4 or 5 after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as representing residue identity. No N- nor C-terminal extensions, deletions nor insertions shall be construed as reducing identity or homology.

PF4AR qualitative biological activity is defined as any one of (1) immunological cross-reactivity with at least one epitope of a polypeptide set forth in Figs. 2, 4, or 5; (2) the ability to specifically bind to a member of the PF4 superfamily; or (3) any effector or functional activity of the Figs. 2, 4 or 5 polypeptides as found in nature, including their ability to bind any ligands other than superfamily members.

Immunologically cross-reactive as used herein means that the candidate polypeptide is capable of competitively inhibiting the binding of a PF4AR to polyclonal antibodies or antisera raised against a PF4AR. Such antibodies and antisera are prepared in conventional fashion by injecting an animal such as a goat or rabbit, for example, subcutaneously with the known native PF4AR in complete Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freund's.

-6-

Included within the scope of the PF4AR as that term is used herein are polypeptides having the amino acid sequences described in Figs. 2, 4 or 5, amino acid sequence variants of such amino acid sequences, glycosylation variants of the polypeptides and covalent modifications of the polypeptides. Each of these are described in more detail below.

5 "Isolated" PF4AR nucleic acid or polypeptide is a PF4AR nucleic acid or polypeptide that is identified and separated from at least one contaminant (nucleic acid or polypeptide respectively) with which it is ordinarily associated in nature, such as from the animal or human source of the PF4AR nucleic acid or polypeptide. In preferred embodiments, the PF4AR will be isolated to pharmaceutically acceptable levels of purity with respect to proteins
10 of its species of origin. In preferred embodiments, PF4AR protein will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by an amino acid sequenator commercially available on the filing date hereof, or (3) to homogeneity by conventional nonreducing SDS-
15 PAGE using Coomassie blue or, preferably, silver stain. Isolated PF4AR includes PF4AR in situ within recombinant cells since, in this instance, at least one component of the PF4AR natural environment will not be present. Isolated PF4AR includes PF4AR from one species in a recombinant cell culture of another species since the receptor in such circumstances will be devoid of source polypeptides. Ordinarily, however, isolated receptor will be prepared by
20 at least one purification step.

Isolated PF4AR nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of the receptor nucleic acid. Isolated PF4AR nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated receptor-encoding
25 nucleic acid includes PF4AR nucleic acid in ordinarily receptor-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature.

The nucleic acid or polypeptide may be labeled for diagnostic and probe purposes, using a label as described and defined further below in the discussion of diagnostic assays.

30 PF4AR "nucleic acid" is defined as RNA or DNA containing greater than ten bases that encodes a polypeptide sequence within Figs. 2, 4 or 5, is complementary to nucleic acid sequence of Figs. 2, 4 or 5, hybridizes to such nucleic acid and remains stably bound to it under low stringency conditions, or encodes a polypeptide sharing at least 30% sequence identity, preferably at least 75%, and more preferably at least 85%, with the translated
35 amino acid sequence shown in Figs. 2, 4 or 5 or a fragment thereof. Preferably the DNA which hybridizes to the nucleic acid of Figs. 2, 4 or 5 contain at least 20, more preferably 40, and more preferably 60 bases. Most preferably, the hybridizing DNA or RNA contains 45 or even more preferably 90 bases. Such hybridizing or complementary nucleic acid, however,

is defined further as being novel and unobvious over any prior art nucleic acid including that which encodes, hybridizes under low stringency conditions, or is complementary to nucleic acid encoding rabbit fMLP receptor⁵, human fMLP receptor *r* (optionally) the IL-8 receptor of Murphy *et al.* (*supra*).

5 "High stringency conditions" are any of those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄ at 50°C; (2) employ during hybridization 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ hybridization
10 with 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Conditions of low stringency are set forth in Example 2.

15 The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

20 Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally,
25 "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

30 The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan. Methods for restriction enzyme digestion, recovery or isolation of DNA, hybridization analysis, and ligation are conventional and by this time well
35 known to the ordinary artisan.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA

-8-

fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, Nucleic Acids Res. 9: 6103-6114 (1981), and Goeddel *et al.*, Nucleic Acids Res. 8:4057 (1980).

5 II. Suitable Methods for Practicing the Invention

1. Preparation of Native PF4AR and Variants

A. Isolation of DNA Encoding PF4AR

The DNA encoding of the PF4AR may be obtained from any cDNA library prepared from tissue believed to contain the PF4AR mRNA, generally HL60 or PBL libraries. The PF4AR gene may also be obtained from a genomic library. Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. We have described the entire cDNA for the IL-8 receptor and two homologous receptors. Nucleic acid encoding this family of receptors is readily obtained under low stringency conditions from genomic DNA or neutrophil cDNA libraries using probes having oligonucleotide sequences from the receptor gene sequences of Figs. 2, 4 or 5. These probes usually will contain about 500 or more bases. Since the probes will hybridize perfectly to the three exemplified DNAs, there is no need to use probe pools containing degenerate sequences. Screening with the probes to identify the Figs. 2, 4 or 5 receptors is more efficient if performed under conditions of high stringency.

Other PF4ARs other than those in Figs. 2, 4 or 5 are believed to exist and to contain regions of homology to the exemplified receptors. Thus probes having the sequences of the DNAs in Figs. 2, 4 or 5 can be used to screen for these receptors as well. The best candidates for probes are long sequences (greater than about 100 bases) that represent sequences that are highly homologous among the three exemplified human receptors. IL-8 cDNA encoding the IL-8 residues 15-34, 78-94, 176-193, 264-282 and 299-312 (and comparable probes from other receptors of the IL-8R family) are useful, particularly in probing for IL-8 receptor DNA. Probes useful for the receptor of Fig. 4 (and isolated proteins characteristic of the Fig. 5 receptor) are represented by sequences comprising residues 1-48, 77-92, 107-137, 156-177, 189-226, 239-257 and 271-315. Homologous probes and residues of the Fig. 4 receptor also are useful, i.e. residues 1-35, 64-78, 94-124, 143-164, 176-197, 219-239 and 251-295. cDNAs comprising cDNA encoding the following regions of the Figs. 2, 4 or 5 polypeptides are useful in probing for other receptors: 92-106, 57-72, 138-154, 314-329 and 57-154.

In general, one first identifies a cell which is capable of specifically binding or which is activated by a given PF4A, typically by *in vitro* bioassays and, optionally, by cell binding analysis using the labelled PF4A. Cells identified by this process (and some are already known for individual PF4As) therefore are expressing a receptor for this PF4A. A cDNA library is prepared from such cells and is screened using the receptor probes by procedures

that are conventional per se. In this instance, however, it is preferred to use low stringency conditions (such as those in Example 2) and then analyze the resulting positive clones for homology to the Figs. 2, 4 or 5 receptors. In general, candidate human PF4ARs will exhibit greater than about 30% amino acid sequence homology to the Figs. 2, 4 or 5 receptors and bear a similar transmembrane loop structure.

Assays are then conducted to confirm that the hybridizing full length genes are the desired PF4AR. The candidate is simply inserted into an expression vector and transformed into a host cell that ordinarily does not bind to the candidate PF4A ligand. Transformants that acquire the ability to bind the ligand thus bear the desired receptor gene. In Example 2, we show that two additional homologous polypeptide sequences representing PR4ARs are identified using IL-8R DNA encoding residues 23-314, although the particular probe is not believed to be critical.

An alternative means to isolate genes encoding additional PF4ARs is to use polymerase chain reaction (PCR) methodology (U.S. Patent 4,683,195; Erlich, ed., PCR Technology, 1989) to amplify the target DNA or RNA, e.g. as described in section 14 of Sambrook *et al.*, supra. This method requires the use of oligonucleotide primers that will be expected to hybridize to the PF4AR, and these readily are selected from the receptor cDNAs of Figs. 2, 4 or 5. Strategies for selection of oligonucleotide primers are described above.

cDNA libraries may be screened from various tissues, preferably mammalian PBL, monocyte, placental, fetal, brain, and carcinoma cell lines in order to obtain DNA encoding the receptors of Figs. 2, 4 or 5, or homologous receptors. More preferably, human or rabbit placental, fetal, brain, and carcinoma cell line cDNA libraries are screened with labelled oligonucleotide probes.

Another method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Engels *et al.* (Agnew. Chem. Int. Ed. Engl. 28:716-734 [1989]). These methods include triester, phosphite, phosphoramidite and H-phosphonate methods, typically proceeding by oligonucleotide synthesis on solid supports. These methods may be used if the entire amino acid or nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. If the desired amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

B. Amino Acid Sequence Variants of the PF4AR

Amino acid sequence variants of the PF4AR are prepared by introducing appropriate nucleotide changes into the PF4AR DNA, or by *in vitro* synthesis of the desired PF4AR polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for the receptors in Figs. 2, 4 or 5. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics.

The amino acid changes also may alter post-translational processing of the PF4AR, such as changing the number or position of glycosylation sites or by altering its membrane anchoring characteristics. Excluded from the scope of this invention are PF4AR variants or polypeptide sequences that are not statutorily novel and unobvious over the prior art.

5 In designing amino acid sequence variants of PF4ARs, the location of the mutation site and the nature of the mutation will depend on the PF4AR characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or
10 a different class adjacent to the located site, or combinations of options 1-3.

A useful method for identification of certain residues or regions of the PF4AR polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science 244:1081-1085 [1989]). Here, a residue or group of target residues are identified (e.g., charged residues such as arg,
15 asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid
20 sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed PF4AR variants are screened for the optimal combination of desired activity.

In general, the regions of the PF4AR molecule preferred for alterations are non-
25 hydrophobic regions or regions that are not highly conserved. Such regions are those in which sequences of 5 or more residues are not substantially conserved in the homologous positions in the rabbit fMLP receptor, the human fMLP receptor, the human C5a receptor and the receptors of Figs. 2, 4 and 5.

PF4AR variants will exhibit at least a biological activity of the parental sequence, for
30 example ligand binding activity or antigenic activity. Antigenically active PF4AR is a polypeptide that binds with an affinity of at least about 10^{-6} 1/mole to an antibody raised against a naturally occurring PF4AR sequence. Ordinarily the polypeptide binds with an affinity of at least about 10^{-6} 1/mole. Most preferably, the antigenically active PF4AR is a polypeptide that binds to an antibody raised against the receptor in its native conformation,
35 "native conformation" generally meaning the receptor as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of the receptor (this can be determined, for example, by migration on nonreducing, nondenaturing sizing gels). Antibody used in determination of antigenic activity

is rabbit polyclonal antibody raised by formulating the native non-rabbit receptor in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-receptor antibody plateaus.

5 One group of variants are deletion mutants, or fragments of the sequences set forth in Figs. 2, 4, 5 or other PF4AR. In general, the fragments are those which constitute the extracellular regions of the receptors (these receptors are unlike most in that they are believed to contain a plurality of hydrophobic, trans-membrane domains separated by hydrophilic sequences believed to loop into the ectoplasm). Particularly of interest are the N-terminal
10 extracellular region containing acidic amino acid residues. However, any sequence which is capable of raising an antibody that will cross-react with the intact receptor, or which will bind to a member of the PF4 superfamily, is useful. These fragments typically will contain a consecutive sequence of at least about 5 (and ordinarily at least about 10) residues.

Amino acid sequence deletions generally range from about 1 to 30 residues, more
15 preferably about 1 to 10 residues, and typically are contiguous. Deletions may be introduced into regions of low homology among the receptors of Figs. 2, 4 and 5 to modify the activity of the receptors. Such deletions will be more likely to modify the biological activity of the receptors more significantly than deletions made elsewhere. The number of consecutive deletions will be selected so as to preserve the tertiary structure of the PF4AR in the affected
20 domain, e.g., beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the PF4AR sequence) may range generally from about 1 to 10 residues,
25 more preferably 1 to 5, most preferably 1 to 3.

Insertional variants of the PF4AR or its extracellular segments include the fusion to the N- or C-terminus of the PF4AR of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions
30 (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the PF4AR molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites
35 identified as the active site(s) of the PF4AR, and sites where the amino acids found in the PF4AR from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity.

-12-

Other sites of interest are those in which particular residues of the PF4ARs of Figs. 2, 4 and 5 are identical. These positions may be important for the biological activity of the PF4AR. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table 1

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
10	Ala (A)	val; leu; ile	val
	Arg (R)	lys; gln; asn	lys
15	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
	Cys (C)	ser	ser
	Gln (Q)	asn	asn
	Glu (E)	asp	asp
20	Gly (G)	pro	pro
	His (H)	asn; gln; lys; arg	arg
	Ile (I)	leu; val; met; ala; phe; norleucine	leu
	Leu (L)	norleucine; ile; val; met; ala; phe	ile
25	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala	leu
	Pro (P)	gly	gly
30	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
35	Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of the PF4AR are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target

-13-

site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- 5 (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of the PF4AR that are homologous with other PF4ARs, or, more preferably, into the non-homologous regions of the molecule.

Any cysteine residues not involved in maintaining the proper conformation of the PF4AR may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the PF4AR is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the PF4AR. These techniques may utilize PF4AR nucleic acid (DNA or RNA), or nucleic acid complementary to the PF4AR nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of PF4AR DNA. This technique is well known in the art, for example as described by Adelman *et al.*, DNA 2:183 (1983). Briefly, the PF4AR DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the PF4AR. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the PF4AR DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea *et al.* (Proc. Natl. Acad. Sci. USA 75:5765 (1978)).

-14-

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the PF4AR, and the other strand (the original template) encodes the native, unaltered sequence of the PF4AR. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. The cells are plated onto agarose plates, and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion. After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding PF4AR mutants at more than one site may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single

-15-

oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

5 In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

10 The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and
15 second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of the PF4AR. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see
20 Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid
25 DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region
30 of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

35 If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant

-16-

primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.* (Gene, 34:315 [1985]).

5 C. Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding native or variant PF4AR is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

15 (i) Signal Sequence Component

In general, a signal sequence may be a component of the vector, or it may be a part of the PF4AR DNA that is inserted into the vector. The native pro PF4AR DNA is directed to the cell surface in our recombinant cells but it does not contain a conventional signal and no N-terminal polypeptide is cleaved during post-translational processing of the polypeptide during membrane insertion of the PF4AR.

20 (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

-17-

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the PF4AR DNA. However, the recovery of genomic DNA encoding the PF4AR is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the PF4AR DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet. 1:327 [1982]), mycophenolic acid (Mulligan *et al.*, Science 209:1422 [1980]) or hygromycin (Sugden *et al.*, Mol. Cell. Biol. 5:410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the PF4AR nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the PF4AR. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the PF4AR are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed

is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the PF4AR. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the PF4AR, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, Nature 282:39 [1979]; Kingsman *et al.*, Gene 7:141 [1979]; or Tschemper *et al.*, Gene 10:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics 85:12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) Promoter Component

Expression vectors usually contain a promoter that is recognized by the host organism and is operably linked to the PF4AR nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the PF4AR, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the PF4AR by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native PF4AR promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the PF4AR DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed PF4AR as compared to the native PF4AR promoter.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang *et al.*, Nature, 275:615 [1978]; and Goeddel *et al.*, Nature, 281:544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8:4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80:21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the PF4AR (Siebenlist *et al.*, Cell, 20:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the PF4AR.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7:149 [1968]; and Holland, Biochemistry, 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

PF4AR transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally

-20-

associated with the PF4AR sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, 273:113 (1978); Mulligan and Berg, *Science*, 209:1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, *Gene*, 18:355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, *Nature*, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, 79:5166-5170 (1982) on expression of the human Interferon β 1 gene in cultured mouse and rabbit cells, and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the PF4AR of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:993 (1981)) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, 3:1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, *Cell*, 33:729 (1983)) as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the PF4AR DNA, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences

-21-

are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the PF4AR. The 3' untranslated regions also include transcription termination sites.

5 Suitable vectors containing one or more of the above listed components and the desired coding and control sequences are constructed by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

10 For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9:309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65:499 (1980).

15 Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the PF4AR. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the
20 expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the PF4AR that have PF4AR-like
25 activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the PF4AR in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293:620-625 (1981); Mantei *et al.*, Nature, 281:40-46 (1979); Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the PF4AR is pRK5 (EP pub. no. 307,247).
30

D. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* χ 1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell
35

-22-

should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors containing PF4AR DNA. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *S. pombe* [Beach and Nurse, Nature, 290:140 (1981)], *Kluyveromyces lactis* [Louvencourt *et al.*, J. Bacteriol., 737 (1983)], *Yarrowia* [EP 402,226], *Pichia pastoris* [EP 183,070], *Trichoderma reesia* [EP 244,234], *Neurospora crassa* [Case *et al.*, Proc. Natl. Acad. Sci. USA, 76:5259-5263 (1979)], and *Aspergillus* hosts such as *A. nidulans* [Ballance *et al.*, Biochem. Biophys. Res. Commun., 112:284-289 (1983); Tilburn *et al.*, Gene, 26:205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81:1470-1474 (1984)] and *A. niger* [Kelly and Hynes, EMBO J., 4:475-479 (1985)].

Suitable host cells for the expression of glycosylated PF4AR polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow *et al.*, BioTechnology, 6:47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, 8:277-279 (Plenum Publishing, 1986), and Maeda *et al.*, Nature, 315:592-594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the PF4AR DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding PF4AR is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the PF4AR DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

-23-

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2).

Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al.*, *supra*, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA

into cells such as by nuclear injection, electroporation, or by protoplast fusion may also be used.

E. Culturing the Host Cells

Prokaryotic cells used to produce the PF4AR polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the PF4AR of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58:44 (1979), Barnes and Sato, Anal. Biochem., 102:255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GentamycinTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

It is further envisioned that the PF4AR of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the PF4AR. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired PF4AR. The control element does not encode the PF4AR of this invention, but the DNA is present in the host cell genome. One next screens for cells making the PF4AR of this invention, or increased or decreased levels of expression, as desired.

F. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes,

-25-

particularly ^{32}P . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75:734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native PF4AR polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

G. Purification of The PF4AR Polypeptide

The PF4AR is recovered from the culture cells by solubilizing cell membrane in detergent.

When a human PF4AR is expressed in a recombinant cell other than one of human origin, the PF4AR is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the PF4AR from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the PF4AR. As a first step, the cells are centrifuged to separate them from culture medium. The membrane and soluble protein fractions are then separated. The PF4AR may then be purified from the membrane fraction of the culture lysate by solubilization with detergents followed by suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; hydrophobic affinity resins and ligand affinity using the appropriate PF4A immobilized on a matrix.

PF4AR variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native PF4AR, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a PF4AR

fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-PF4AR column can be employed to absorb the PF4AR variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native PF4AR may require modification to account for changes in the character of the PF4AR or its variants upon expression in recombinant cell culture.

H. Covalent Modifications of PF4AR Polypeptides

Covalent modifications of PF4AR polypeptides are included within the scope of this invention. Both native PF4ARs and amino acid sequence variants of the PF4AR may be covalently modified. Covalent modifications of the PF4AR, fragments thereof or antibodies thereto are introduced into the molecule by reacting targeted amino acid residues of the PF4AR, fragments thereof, or PF4AR antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues. Most commonly, PF4AR and its antibodies are covalently bonded to detectable groups used in diagnosis, e.g. enzymes, radio isotopes, spin labels, antigens, fluorescent or chemiluminescent groups and the like.

Cysteiny l residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazole)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysiny l and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny l residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminas e-catalyz ed reaction with glyoxylate.

-27-

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking PF4AR, its fragments or antibodies to a water-insoluble support matrix or surface for use in methods for purifying anti-PF4AR antibodies, and vice versa. Immobilized PF4AR also is useful in screening for the PF4 superfamily members to which the receptor binds. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the beta-8 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native receptor, and/or adding one or more glycosylation sites that are not present in the native receptor.

5 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-
10 peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. As noted above, the OL-8 receptor contains 6 putative N-linked glycosylation sites.

15 Addition of glycosylation sites to the PF4AR polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native PF4AR sequence (for O-linked glycosylation sites). For ease, the PF4AR amino acid sequence
20 is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the PF4AR polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of PF4AR Polypeptide".

25 Another means of increasing the number of carbohydrate moieties on the PF4AR polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- and O- linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free
30 carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston (CRC Crit. Rev. Biochem., pp. 259-306 [1981]).

35 Removal of carbohydrate moieties present on the native PF4AR polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-

acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin *et al.* (Arch. Biochem. Biophys., 259:52 [1987]) and by Edge *et al.* (Anal. Biochem., 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.* (Meth. Enzymol., 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.* (J. Biol. Chem., 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

The PF4AR also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacrylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

PF4AR preparations are also useful in generating antibodies, for use as standards in assays for the PF4AR (e.g. by labeling the PF4AR for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant PF4AR, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the PF4AR molecule, such as affinity for a given antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for native PF4AR in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

3. Therapeutic Compositions and Administration of PF4AR

Therapeutic formulations of PF4AR (including its PF4AR binding fragments) or antibodies thereto are prepared for storage by mixing PF4AR having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, *supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine,

asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

5 The PF4AR, or antibody to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The PF4AR ordinarily will be stored in lyophilized form or in solution.

10 Therapeutic PF4AR or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of PF4AR or antibody administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below.

15 Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22:547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (Langer *et al.*, J. Biomed. Mater. Res., 15:167-
20 277 [1981] and Langer, Chem. Tech., 12:98-105 [1982]), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release PF4AR or antibody compositions also include liposomally entrapped PF4AR or antibody. Liposomes containing PF4AR or antibody are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82:3688-3692 (1985); Hwang *et al.*, Proc. Natl.
25 Acad. Sci. USA, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal PF4AR or antibody therapy.

30 An effective amount of PF4AR or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the PF4AR or antibody until a dosage is reached that
35 achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

4. PF4AR Antibody Preparation

Polyclonal antibodies to the PF4AR generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the PF4AR and an adjuvant. Immunization with recombinant cells transformed with the PF4AR (e.g. mouse or CHO cells transformed with huPF4AR) may be satisfactory, or it may be useful to separate the PF4AR and conjugate it or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N} = \text{C} = \text{NR}$, where R and R^1 are different alkyl groups.

Animals ordinarily are immunized against the cells or immunogenic conjugates or derivatives by combining 1 mg or 1 μg of PF4AR of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-PF4AR titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same PF4AR, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Another option is to employ combinatorial variable domain libraries and screening methods to identify the desired anti-PF4AR antibodies.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody.

The monoclonal antibody preferably is specific for each target PF4AR polypeptide, and will not cross-react with rabbit fMLP receptor⁵, human fMLP receptor, human C5a receptor the low affinity IL-8 receptor or other members of the PF4AR family. Antibodies specific for the receptor of Figs. 2, 4 or 5 are preferred. The antibody is selected to be either agonistic, antagonistic or to have no effect on the activity of a PF4 super-family member in binding to or activating the receptor.

Murphy et al. (*supra*) describe a receptor having a high degree of homology to the receptor of Fig. 2. The Murphy et al. characterized their receptor in recombinant oocytes as being a "low affinity" receptor for IL-8 and having little capability to bind MGSA, thus suggesting that it plays a minor role in IL-8 and MGSA biological activity *in vivo*. Our studies, however, have shown that the Murphy et al. receptor exhibits IL-8 affinity as high or higher

-32-

than the receptor of Fig. 2 and that as well it shows high affinity (about 1-10nM) for MGSA. Thus, antagonism of the IL-8 and/or MGSA response of lymphoid cells will likely require that both receptors be inhibited or blocked. For example, one should select an IL-8 antagonist antibody that binds to an epitope of the Fig. 2 receptor that is shared by the Murphy et al. receptor. This could be readily accomplished by routine screening methods. For example, the candidate antibodies can be assayed for their ability to compete against labelled IL-8 for binding to cells bearing the Fig. 2 receptor, and then the same study conducted with cells bearing the Murphy et al. receptor. Antibodies that inhibit IL-8 activation or binding to both cells are then selected as therapeutic candidates. On the other hand, antibodies that can discriminate between the Fig. 2 and Murphy et al. receptors and bind only to one or the other are useful in diagnosis. The receptor of Fig. 2 binds MGSA poorly, in contrast to the Murphy et al. receptor.

5. Uses of PF4AR its nucleic acid and its Antibodies

The nucleic acid encoding the PF4AR may be used as a diagnostic for tissue specific typing. For example, such procedures as *in situ* hybridization, and northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding the PF4AR are present in the cell type(s) being evaluated. These receptors typically are diagnostic of PBL or monocytic cells.

Isolated PF4AR polypeptide may be used in quantitative diagnostic assays as a standard or control against which samples e.g. from PBL or monocytic cells, containing unknown quantities of PF4AR may be compared. Recombinant cells which express the IL-8 receptor can be used in assays for PF4A ligands in the same fashion as for example neutrophils are used in IL-8 assays. The PF4AR polypeptides, fragments or cells (as such, or derivatized) also can be used as immunogens in the production of antibodies to PF4AR, for the purification of such antibodies from ascites or recombinant cell culture media or for use as competitive antagonists for superfamily ligands, e.g. IL-8.

The PF4AR are useful in screening for amino acid sequence or other variants of PF4 superfamily members. For example, a bank of candidate IL-8 amino acid sequence variants are prepared by site directed mutagenesis. These are incubated in competition with labelled native IL-8 for cells bearing the IL-8 receptor of Fig. 2 in order identify agonist or antagonist IL-8 variants. Binding or cell activation are suitable assay endpoints. Alternatively, the receptor is recovered in cell-free form and binding of IL-8 and candidate variants assayed.

PF4AR antibodies are useful in diagnostic assays for PF4AR expression in specific cells or tissues wherein the antibodies are labeled in the same fashion as the PF4AR described above and/or are immobilized on an insoluble matrix. PF4AR antibodies also are useful for the affinity purification of the PF4AR from recombinant cell culture or natural sources. The PF4AR antibodies that do not detectably cross-react with other PF4ARs can be used to purify each PF4AR free from other homologous receptors. PF4AR antibodies that are PF4

antagonists are useful as anti-inflammatory agents or in the treatment of other PF4 superfamily-mediated disorders.

Suitable diagnostic assays for the PF4AR and its antibodies are well known *per se*. Such assays include competitive and sandwich assays, and steric inhibition assays.

5 Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the PF4AR and for substances that bind the PF4AR, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to
10 herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the PF4AR or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner,
15 immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label PF4AR nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including
20 moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{131}I , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase
25 (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or
30 microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the
35 antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter *et al.*, Nature, 144:945 (1962); David *et al.*, Biochemistry, 13:1014-1021 (1974); Pain *et al.*, J. Immunol. Methods, 40:219-230 (1981); and Nygren, J. Histochem. and Cytochem., 30:407-

412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

5 The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan *et al.*, "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166. Such bonding methods are suitable for use with PF4AR or its antibodies, all of which are proteinaceous.

10 Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al.*, U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

15 Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

20 Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

25 Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the PF4AR or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with anti-PF4AR so that binding of the anti-PF4AR inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of
35 EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same

time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

5 Sandwich assays particularly are useful for the determination of PF4AR or PF4AR antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-PF4AR monoclonal antibody as one antibody and a polyclonal anti-PF4AR antibody as the other is useful in testing samples for PF4AR activity.

The foregoing are merely exemplary diagnostic assays for PF4AR and antibodies. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

The polypeptides set forth in Figs. 4 and 5 are believed to represent receptors for different and as yet undetermined members of the PF4 superfamily (which includes both the C-C and CXC subfamilies). Like the IL-8 receptor of Fig. 2 they are members of the G-protein-coupled superfamily and bear greater similarity to the IL-8 receptor than other receptors. In preliminary experiments, recombinant cells bearing these receptors do not respond to Rantes, MCP1, IL-8 or MGSA, although they may ultimately be shown to bind other members of the PF4 superfamily or presently unknown ligands. However, whether or not the Figs. 4 or 5 polypeptides bind to members of the PF4 superfamily, the polypeptides are useful for preparing antibodies for diagnostic use in determining the tissue distribution of the receptors and thus as an immunohistochemical diagnostic for such tissues, in particular as a diagnostic for monocytic cells or PBLs since it is known that such cells express the receptors of Figs. 4 and 5. Of course, once the PF4 superfamily members are identified which bind to these receptors then the receptors can be used to diagnose the presence of the identified members or for their purification in specific affinity procedures. The DNA in Figs. 4 and 5 also is useful in diagnostics for the presence of DNA or RNA encoding the IL-8 receptor when low stringency conditions are employed.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

To obtain the clone pRK5B.il8r1.1, a cDNA¹⁶ library of 1,000,000 clones was constructed from human neutrophil mRNA¹⁷ in the vector pRK5B using bstXI linkers. The cDNA is produced in blunted form. Hemi-kinase bstXI linkers are ligated to the cDNA, and the linkers ligated into the pRK5B vector that had been bstXI digested, phosphatased, and the long vector fragment isolated. pRK5B is a derivative of pRK5¹⁸ that contains a cytomegalovirus

-36-

promoter followed by a 5' intron, bstXI cloning site, and an SV40 early p lyadenylation signal, although it will be understood that any mammalian cell expression vector will be satisfactory. 58 pools of 2500 clones each were transfected into COS-7 cells by electroporation¹⁹ of 20 μ g of DNA into 3,750,000 cells. After 2 days of growth on 150-mm dishes in medium (50:50::Ham's F12:DMEM) containing 10 % fetal calf serum, ¹²⁵I-IL-8 binding was performed. Purified human 72 amino acid IL-8 made in *E. coli*²⁰ was labeled by the lactoperoxidase method²¹ to about 1100 Ci/mmol and was at least 85 % bindable. Dishes were rinsed twice with phosphate-buffered saline, and binding was performed with 8 ml per dish of growth medium containing 2.5 % fetal calf serum and about 0.5 nM ¹²⁵I-IL-8. After 2 hr at 37°, the plates were rinsed three times with phosphate-buffered saline, the bottoms cut out²² and autoradiographed. Each positive pool of 2500 cDNA clones was subsequently partitioned into pools of 800 clones, and each of these was transfected and assayed. Each positive pool in turn was subdivided through pools of 185, 30 and finally a single clone(s) until single positive clones were identified to obtain the pure isolate. Since only a portion of each pool was used for transfection it was unnecessary to rescue clones from transformants.

Binding competition was performed with electroporated COS-7 cells after 1 day of expression in 6-well dishes (about 175,000 cells/dish). Binding was performed with radioiodinated wild type IL-8 in binding medium Ca²⁺ and Mg²⁺-free Hanks buffered with 25 nM HEPES and supplemented with 0.5% BSA) at 4° for about 2 hr. Wells were then washed, the cells harvested with trypsin, and counted. No specific binding was found in parallel wells containing cells transfected with DNA from the vector pRK5B. Neutrophil binding was performed as described²² but for 2 hr at 4°.

EXAMPLE 2

Existing λ gt10 cDNA libraries from the human cell line, HL60, and from human peripheral blood lymphocytes were screened at low stringency with a probe from the coding region of the cloned high-affinity human IL-8 receptor (Fig. 2). The probe was the 874 bp PstI/NcoI fragment of the receptor containing the coding region for amino acids 23-314. Hybridization was in 20% formamide, 4 x SSC, 50 mM sodium phosphate buffer, pH 7, 0.2 g/l sonicated salmon sperm DNA, 5 x Denhardt's, 10% dextran sulfate, at 42°C with a wash at 1 x SSC, 0.1% SDS at 50°C. A number of duplicate spots of varying intensity (about 60) were picked, plaque purified, subcloned into plasmid vectors, and sequenced. Nucleic acid sequencing began with the selection of spots of greatest intensity. Sufficient sequence was obtained for a given spot (phage) to determine whether or not evidence of structural or sequence homology with the IL-8 receptor existed. If it did, then the remainder of the gene was obtained (if necessary) and sequenced in its entirety. To avoid sequences all hybridizing clone the sequence was then used to probe the parental collection of IL-8 receptor DNA hybridizing clones under high stringency conditions in order to identify and discard other

-37-

spots containing the same hybridizing gene. This technique was highly effective in reducing the sequencing burden. For example, one clone was represented by about one third of the initial 60 clones, and on this result alone the negative screen was able to reduce considerably the work involved in sequencing the clones.

- 5 From this screen, two new gene sequences were found that are clearly related to the IL-8 receptor. The coding region for one new gene was split between two clones (8rr.20 and 8rr.15). The combined sequence of this gene (8rr.20.15) is shown in Figure 4. The complete coding region for the second gene is found on clone 8rr.9 (Figure 5). The predicted amino acid sequence of 8rr.20.15 is 34% identical with both the high and low affinity IL-8 receptor sequences. The sequence of 8rr.9 is 36% and 38% identical with the high and low affinity IL-8 receptor sequences, respectively (W.E. Holmes *et al.*, Science 253, 1278 (1991) and P.M. Murphy *et al.*, Science 253, 1280 (1991). The amino acid sequence of 8rr.20.15 and 8rr.9 are 31% identical. Use of this probe under low stringency conditions did not produce detectable hybridization to the fMLP receptor genes that were expected to be found in these
- 10
- 15 libraries.

References

1. Oppenheim, J.J. *et al.*, Annu. Rev. Immunol., 9:617-648 (1991).
2. Gimbrone, M.A.Jr. *et al.*, Science, 246:1601-3 (1989).
3. Boulay, F. *et al.*, Biochem. Biophys. Res. Comm., 168:1103-1109 (1990).
- 5 4. Gerard, N.P. & Gerard, C., Nature, 349:614-617 (1991).
5. Thomas, K.M., Pyun, H.Y. & Navarro, J., J. Biol. Chem., 265:20061-20064 (1990).
6. Sims, J.E. *et al.*, Science, 241:585-589 (1988).
7. D'Andrea, A.D., Lodish, H.F. & Wong, G.G., Cell, 57:277-285 (1989).
8. Samanta, A.K., Oppenheim, J.J. & Matsushima, K., J. Exp. Med., 169:1185-1189
10 (1989).
9. Besemer, J., Hujber, A. & Kuhn, B., J. Biol. Chem., 264:17409-17415 (1989).
10. Grob, P.M. *et al.* J. Biol. Chem., 265:8311-8316 (1990).
11. Kozak, M., Nucleic Acid Res., 12:857-872 (1984).
12. Dixon, R.A.F., Sigal, I.S. & Strader, C.D., Cold Spring Har. Sym. Quant. Biol.,
15 53:487-497 (1988).
13. Korchak, H.M. *et al.*, J. Biol. Chem., 259:4076-4082 (1984).
14. Tennenberg, S.D., Zemlan, F.P. & Solomkin J.S., J. Immunol., 141:3937-3944 (1988).
15. Ramachandran, J. *et al.*, BioEssays, 10:54-57 (1989).
16. Gubler, U. & Hoffman, B.J. Gene, 25, 263-269, (1983).
- 20 17. Chirgwin, J.J. *et al.* Biochem., 18:5294-5299 (1979).
18. EP 307,247.
19. Gearing, D.P. *et al.* EMBO J., 8:3667-3676 (1989).
20. Hebert, C.A. *et al.* J. Immunol., 145:3033-3040 (1991).
21. Morrison M. & Bayse, G.S., Biochem., 9:2995-3000 (1970).
- 25 22. Pacholczyk, T., Blakely, R.D. & Amara, S.G., BioTechniques, 9:556-558 (1990).
23. Gryniewicz, G., Poenie, M. & Tsien, R.Y., J. Biol. Chem., 260:3440-3450 (1985).

-39-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: GENENTECH, INC.
- (ii) TITLE OF INVENTION: Human PF4A Receptors and Their Use
- 10 (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 (A) ADDRESSEE: Genentech, Inc.
 (B) STREET: 460 Point San Bruno Blvd
 (C) CITY: South San Francisco
 15 (D) STATE: California
 (E) COUNTRY: USA
 (F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
 20 (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: patin (Genentech)
- 25 (vi) CURRENT APPLICATION DATA:
 (A) APPLICATION NUMBER:
 (B) FILING DATE: 23-Mar-1992
 (C) CLASSIFICATION:
- 30 (vii) PRIOR APPLICATION DATA:
 (A) APPLICATION NUMBER: 07/677211
 (B) APPLICATION DATE: 29-Mar-1991
- (viii) PRIOR APPLICATION DATA:
 35 (A) APPLICATION NUMBER: 07/810782
 (B) APPLICATION DATE: 19-Dec-1991
- (viii) ATTORNEY/AGENT INFORMATION:
 40 (A) NAME: Hensley, Max D.
 (B) REGISTRATION NUMBER: 27,043
 (C) REFERENCE/DOCKET NUMBER: 706P1
- (ix) TELECOMMUNICATION INFORMATION:
 45 (A) TELEPHONE: 415/266-1994
 (B) TELEFAX: 415/952-9881
 (C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1933 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 55 (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

60 CCTGGCCGGT GCTTCAGTTA GATCAAACCA TTGCTGAAAC TGAAGAGGAC 50

ATG TCA AAT ATT ACA GAT CCA CAG ATG TGG GAT TTT 86
 Met Ser Asn Ile Thr Asp Pro Gln Met Trp Asp Phe
 1 5 10

65 GAT GAT CTA AAT TTC ACT GGC ATG CCA CCT GCA GAT GAA 125
 Asp Asp Leu Asn Phe Thr Gly Met Pro Pro Ala Asp Glu
 15 20 25

-40-

	GAT	TAC	AGC	CCC	TGT	ATG	CTA	GAA	ACT	GAG	ACA	CTC	AAC	164
	Asp	Tyr	Ser	Pro	Cys	Met	Leu	Glu	Thr	Glu	Thr	Leu	Asn	
					30					35				
5	AAG	TAT	GTT	GTG	ATC	ATC	GCC	TAT	GCC	CTA	GTG	TTC	CTG	203
	Lys	Tyr	Val	Val	Ile	Ile	Ala	Tyr	Ala	Leu	Val	Phe	Leu	
		40					45					50		
10	CTG	AGC	CTG	CTG	GGA	AAC	TCC	CTG	GTG	ATG	CTG	GTC	ATC	242
	Leu	Ser	Leu	Leu	Gly	Asn	Ser	Leu	Val	Met	Leu	Val	Ile	
				55					60					
15	TTA	TAC	AGC	AGG	GTC	GGC	CGC	TCC	GTC	ACT	GAT	GTC	TAC	281
	Leu	Tyr	Ser	Arg	Val	Gly	Arg	Ser	Val	Thr	Asp	Val	Tyr	
		65				70					75			
20	CTG	CTG	AAC	CTG	GCC	TTG	GCC	GAC	CTA	CTC	TTT	GCC	CTG	320
	Leu	Leu	Asn	Leu	Ala	Leu	Ala	Asp	Leu	Leu	Phe	Ala	Leu	
			80					85					90	
25	ACC	TTG	CCC	ATC	TGG	GCC	GCC	TCC	AAG	GTG	AAT	GGC	TGG	359
	Thr	Leu	Pro	Ile	Trp	Ala	Ala	Ser	Lys	Val	Asn	Gly	Trp	
					95					100				
30	ATT	TTT	GGC	ACA	TTC	CTG	TGC	AAG	GTG	GTC	TCA	CTC	CTG	398
	Ile	Phe	Gly	Thr	Phe	Leu	Cys	Lys	Val	Val	Ser	Leu	Leu	
		105					110					115		
35	AAG	GAA	GTC	AAC	TTC	TAC	AGT	GGC	ATC	CTG	CTG	TTG	GCC	437
	Lys	Glu	Val	Asn	Phe	Tyr	Ser	Gly	Ile	Leu	Leu	Leu	Ala	
				120					125					
40	TGC	ATC	AGT	GTG	GAC	CGT	TAC	CTG	GCC	ATT	GTC	CAT	GCC	476
	Cys	Ile	Ser	Val	Asp	Arg	Tyr	Leu	Ala	Ile	Val	His	Ala	
		130				135					140			
45	ACA	CGC	ACA	CTG	ACC	CAG	AAG	CGT	CAC	TTG	GTC	AAG	TTT	515
	Thr	Arg	Thr	Leu	Thr	Gln	Lys	Arg	His	Leu	Val	Lys	Phe	
			145					150					155	
50	GTT	TGT	CTT	GGC	TGC	TGG	GGA	CTG	TCT	ATG	AAT	CTG	TCC	554
	Val	Cys	Leu	Gly	Cys	Trp	Gly	Leu	Ser	Met	Asn	Leu	Ser	
					160					165				
55	CTG	CCC	TTC	TTC	CTT	TTC	CGC	CAG	GCT	TAC	CAT	CCA	AAC	593
	Leu	Pro	Phe	Phe	Leu	Phe	Arg	Gln	Ala	Tyr	His	Pro	Asn	
					170			175				180		
60	AAT	TCC	AGT	CCA	GTT	TGC	TAT	GAG	GTC	CTG	GGA	AAT	GAC	632
	Asn	Ser	Ser	Pro	Val	Cys	Tyr	Glu	Val	Leu	Gly	Asn	Asp	
				185					190					
65	ACA	GCA	AAA	TGG	CGG	ATG	GTG	TTG	CGG	ATC	CTG	CCT	CAC	671
	Thr	Ala	Lys	Trp	Arg	Met	Val	Leu	Arg	Ile	Leu	Pro	His	
					200						205			
70	ACC	TTT	GGC	TTC	ATC	GTG	CCG	CTG	TTT	GTC	ATG	CTG	TTC	710
	Thr	Phe	Gly	Phe	Ile	Val	Pro	Leu	Phe	Val	Met	Leu	Phe	
			210					215					220	
75	TGC	TAT	GGA	TTC	ACC	CTG	CGT	ACA	CTG	TTT	AAG	GCC	CAC	749
	Cys	Tyr	Gly	Phe	Thr	Leu	Arg	Thr	Leu	Phe	Lys	Ala	His	
					225					230				
80	ATG	GGG	CAG	AAG	CAC	CGA	GCC	ATG	AGG	GTC	ATC	TTT	GCT	788
	Met	Gly	Gln	Lys	His	Arg	Ala	Met	Arg	Val	Ile	Phe	Ala	
		235					240					245		

-41-

GTC GTC CTC ATC TTC CTG CTT TGC TGG CTG CCC TAC AAC 827
 Val Val Leu Ile Phe Leu Leu Cys Trp Leu Pro Tyr Asn
 250 255

5 CTG GTC CTG CTG GCA GAC ACC CTC ATG AGG ACC CAG GTG 866
 Leu Val Leu Leu Ala Asp Thr Leu Met Arg Thr Gln Val
 260 265 270

10 ATC CAG GAG ACC TGT GAG CGC CGC AAC AAC ATC GGC CGG 905
 Ile Gln Glu Thr Cys Glu Arg Arg Asn Asn Ile Gly Arg
 275 280 285

15 CCC CTG GAT GCC ACT GAG ATT CTG GGA TTT CTC CAT AGC 944
 Ala Leu Asp Ala Thr Glu Ile Leu Gly Phe Leu His Ser
 290 295

20 TGC CTC AAC CCC ATC ATC TAC GCC TTC ATC GGC CAA AAT 983
 Cys Leu Asn Pro Ile Ile Tyr Ala Phe Ile Gly Gln Asn
 300 305 310

TTT CGC CAT GGA TTC CTC AAG ATC CTG GCT ATG CAT GGC 1022
 Phe Arg His Gly Phe Leu Lys Ile Leu Ala Met His Gly
 315 320

25 CTG GTC AGC AAG GAG TTC TTG GCA CGT CAT CGT GTT ACC 1061
 Leu Val Ser Lys Glu Phe Leu Ala Arg His Arg Val Thr
 325 330 335

30 TCC TAC ACT TCT TCG TCT GTC AAT GTC TCT TCC AAC CTC 1100
 Ser Tyr Thr Ser Ser Ser Val Asn Val Ser Ser Asn Leu
 340 345 350

TGA AAACCAT CGATGAAGGA ATATCTCTTC TCAGAAGGAA AGAATAACCA 1150

35 ACACCCTGAG GTTGTGTGTG GAAGGTGATC TGGCTCTGGA CAGGCACTAT 1200

40 CTGGGTTTTG GGGGGACGCT ATAGGATGTG GGGAGTTAG GAACTGGTGT 1250

CTT CAGGGGC CACACCAACC TTCTGAGGAG CTGTTGAGGT ACCTCCAAGG 1300

45 ACCGGCCTTT GCACCTCCAT GGAAACGAAG CACCATCATT CCGTTGAAC 1350

GTCACATCTT TAACCCACTA ACTGGCTAAT TAGCATGGCC ACATCTGAGC 1400

50 CCCGAATCTG ACATTAGATG AGAGAACAGG GCTGAAGCTG TGTCTCATG 1450

55 AGGGCTGGAT GCTCTCGTTG ACCCTCAGAG GAGCATCTCC TCAACTCTGA 1500

GTGTTAAGCG TTGAGCCACC AAGCTGGTGG CTCTGTGTGC TCTGATCCGA 1550

60 GCTCAGGGGG GTGGTTTTCC CATCTCAGGT GTGTTGCAGT GTCTGCTGGA 1600

GACATTGAGG CAGGCACTGC CAAAACATCA ACCTGCCAGC TGGCCTTGTG 1650

65 AGGAGCTGGA AACACATGTT CCCCTTGGGG GTGGTGGATG AACAAAGAGA 1700

-42-

5 AAGAGGGTTT GGAAGCCAGA TCTATGCCAC AAGAACCCCC TTTACCCCCA 1750
 TGACCAACAT CGCAGACACA TGTGCTGGCC ACCTGCTGAG CCCCAAGTGG 1800
 AACGAGACAA GCAGCCCTTA GCCCTTCCCC TCTGCAGCTT CCAGGCTGGC 1850
 10 GTGCAGCATC AGCATCCCTA GAAAGCCATG TGCAGCCACC AGTCCATTGG 1900
 GCAGGCAGAT GTTCCTAATA AAGCTTCTGT TCC 1933

15

(2) INFORMATION FOR SEQ ID NO:2:

(1) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 1737 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30 GAATTCCAGT GTGCTGGCGG CGCGGCGCAA AGTGACGCCG AGGGCCTGAG 50
 TGCTCCAGTA GCCACCGCAT CTGGAGAACC AGCGGTTACC ATG GAG 96
 Met Glu
 1
 35 GGG ATC AGT ATA TAC ACT TCA GAT AAC TAC ACC GAG GAA 135
 Gly Ile Ser Ile Tyr Thr Ser Asp Asn Tyr Thr Glu Glu
 5 10 15
 40 ATG GGC TCA GGG GAC TAT GAC TCC ATG AAG GAA CCC TGT 174
 Met Gly Ser Gly Asp Tyr Asp Ser Met Lys Glu Pro Cys
 20 25
 45 TTC CGT GAA GAA AAT GCT AAT TTC AAT AAA ATC TTC CTG 213
 Phe Arg Glu Glu Asn Ala Asn Phe Asn Lys Ile Phe Leu
 30 35 40
 CCC ACC ATC TAC TCC ATC ATC TTC TTA ACT GGC ATT GTG 252
 Pro Thr Ile Tyr Ser Ile Ile Phe Leu Thr Gly Ile Val
 45 50
 50 GGC AAT GGA TTG GTC ATC CTG GTC ATG GGT TAC CAG AAG 291
 Gly Asn Gly Leu Val Ile Leu Val Met Gly Tyr Gln Lys
 55 60 65
 55 AAA CTG AGA AGC ATG ACG GAC AAG TAC AGG CTG CAC CTG 330
 Lys Leu Arg Ser Met Thr Asp Lys Tyr Arg Leu His Leu
 70 75 80
 60 TCA GTG GCC GAC CTC CTC TTT GTC ATC ACG CTT CCC TTC 369
 Ser Val Ala Asp Leu Leu Phe Val Ile Thr Leu Pro Phe
 85 90
 TGG GCA GTT GAT GCC GTG GCA AAC TGG TAC TTT GGG AAC 408
 Trp Ala Val Asp Ala Val Ala Asn Trp Tyr Phe Gly Asn
 95 100 105
 65 TTC CTA TGC AAG GCA GTC CAT GTC ATC TAC ACA GTC AAC 447
 Phe Leu Cys Lys Ala Val His Val Ile Tyr Thr Val Asn

-43-

		110		115	
5	CTC TAC AGC AGT GTC CTC ATC CTG GCC TTC ATC AGT CTG 486				
	Leu Tyr Ser Ser Val Leu Ile Leu Ala Phe Ile Ser Leu				
	120	125		130	
10	GAC CGC TAC CTG GCC ATC GTC CAC GCC ACC AAC AGT CAG 525				
	Asp Arg Tyr Leu Ala Ile Val His Ala Thr Asn Ser Gln				
	135	140		145	
15	AGG CCA AGG AAG CTG TTG GCT GAA AAG GTG GTC TAT GTT 564				
	Arg Pro Arg Lys Leu Leu Ala Glu Lys Val Val Tyr Val				
	150			155	
20	GGC GTC TGG ATC CCT GCC CTC CTG CTG ACT ATT CCC GAC 603				
	Gly Val Trp Ile Pro Ala Leu Leu Leu Thr Ile Pro Asp				
	160	165		170	
25	TTC ATC TTT GCC AAC GTC AGT GAG GCA GAT GAC AGA TAT 642				
	Phe Ile Phe Ala Asn Val Ser Glu Ala Asp Asp Arg Tyr				
	175			180	
30	ATC TGT GAC CGC TTC TAC CCC AAT GAC TTG TGG GTG GTT 681				
	Ile Cys Asp Arg Phe Tyr Pro Asn Asp Leu Trp Val Val				
	185	190		195	
35	GTG TTC CAG TTT CAG CAC ATC ATG GTT GGC CTT ATC CTG 720				
	Val Phe Gln Phe Gln His Ile Met Val Gly Leu Ile Leu				
	200	205		210	
40	CCT GGT ATT GTC ATC CTG TCC TGC TAT TGC ATT ATC ATC 759				
	Pro Gly Ile Val Ile Leu Ser Cys Tyr Cys Ile Ile Ile				
	215			220	
45	TCC AAG CTG TCA CAC TCC AAG GGC CAC CAG AAG CGC AAG 798				
	Ser Lys Leu Ser His Ser Lys Gly His Gln Lys Arg Lys				
	225	230		235	
50	GCC CTC AAG ACC ACA GTC ATC CTC ATC CTG GCT TTC TTC 837				
	Ala Leu Lys Thr Thr Val Ile Leu Ile Leu Ala Phe Phe				
	240	245			
55	GCC TGT TGG CTG CCT TAC TAC ATT GGG ATC AGC ATC GAC 876				
	Ala Cys Trp Leu Pro Tyr Tyr Ile Gly Ile Ser Ile Asp				
	250	255		260	
60	TCC TTC ATC CTC CTG GAA ATC ATC AAG CAA GGC TGT GAG 915				
	Ser Phe Ile Leu Leu Glu Ile Ile Lys Gln Gly Cys Glu				
	265	270		275	
65	TTT GAG AAC ACT GTG CAC AAG TGG ATT TCC ATC ACC GAG 954				
	Phe Glu Asn Thr Val His Lys Trp Ile Ser Ile Thr Glu				
	280			285	
70	GCC CTA GCT TTC TTC CAC TGT TGT CTG AAC CCC ATC CTC 993				
	Ala Leu Ala Phe Phe His Cys Cys Leu Asn Pro Ile Leu				
	290	295		300	
75	TAT GCT TTC CTT GGA GCC AAA TTT AAA ACC TCT GCC CAG 1032				
	Tyr Ala Phe Leu Gly Ala Lys Phe Lys Thr Ser Ala Gln				
	305	310			
80	CAC GCA CTC ACC TCT GTG AGC AGA GGC TCC AGC CTC AAG 1071				
	His Ala Leu Thr Ser Val Ser Arg Gly Ser Ser Leu Lys				
	315	320		325	
85	ATC CTC TCC AAA GGA AAG CGA GGT GGA CAT TCA TCT GTT 1110				
	Ile Leu Ser Lys Gly Lys Arg Gly Gly His Ser Ser Val				

-44-

330 335 340

TCC ACT GAG TCT GAG TCT TCA AGT TTT CAC TCC AGC TAAC 1150
 Ser Thr Glu Ser Glu Ser Ser Ser Phe His Ser Ser
 345 350 352

5 ACAGATGTAA AAGACTTTTT TTTATACGAT AAATAACTTT TTTTAAAGTT 1200

10 ACACATTTTT CAGATATAAA AGACTGACCA ATATTGTACA GTTTTTATTG 1250

CTTGTTGGAT TTTTGTCTTG TGTTTCTTTA GTTTTGTGA AGTTTAATTG 1300

15 ACTTATTAT ATAAATTTTT TTTGTTTCAT ATTGATGTGT GTCTAGGCAG 1350

GACCTGTGGC CAACTTCTTA GTTGCTGTAT GTCTCGTGGT AGGACTGTAG 1400

20 AAAAGGGAAC TGAACATTCC AGAGCGTGTA GTGAATCACG TAAAGCTAGA 1450

AATGATCCCC AGCTGTTTAT GCATAGATAA TCTCTCCATT CCCGTGGAAC 1500

25 GTTTTTCCTG TTCTTAAGAC GTGATTTTGC TGTAAGAT GGCACCTATA 1550

30 ACCAAAGCCC AAAGTGGTAT AGAAATGCTG GTTTTTCAGT TTTCAGGAGT 1600

GGGTGATT CAGCACCTAC AGTGACAGT CTTGTATTAA GTTGTAATA 1650

35 AAAGTACATG TTAACTTAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1700

40 AAAAAAAAAA AAAGCGGCCG CCAGCACACT GGAATTC 1737

(2) INFORMATION FOR SEQ ID NO:3:

45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1679 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

55

GAATCCAGT GTGCTGGCGG CCGCCAGTG TGCTGGCGGC GGCAGTTGAG 50

GGAAAGGACA GAGGTATGA GTGCCTGCAA GAGTGGCAGC CTGGAGTAGA 100

60

GAAAACACTA AAGGTGGAGT CAAAGACCT GAGTTCAAGT CCCAGCTCTG 150

CCACTGGTTA GCTGTGGGAT CTCGAAAAG ACCCAGTGAA AAAAAAAAAA 200

65

AAAGTGATGA GTTGTGAGGC AGGTGCGGC CCTACTGCCT CAGGAGACGA 250

-45-

TGGCGAGCTC ATTTGCTTAA ATTTGCAGCT GACGGCTGCC ACCTCTCTAG 300
 AGGCACCTGG CGGGGAGCCT CTCAACATAA GACAGTGACC AGTCTGGTGA 350
 5
 CTCACAGCCG GCACAGCC ATG AAC TAC CCG CTA ACG CTG GAA 392
 Met Asn Tyr Pro Leu Thr Leu Glu
 1 5
 10
 ATG GAC CTC GAG AAC CTG GAG GAC CTG TTC TGG GAA CTG 431
 Met Asp Leu Glu Asn Leu Glu Asp Leu Phe Trp Glu Leu
 10 15 20
 15
 GAC AGA TTG GAC AAC TAT AAC GAC ACC TCC CTG GTG GAA 470
 Asp Arg Leu Asp Asn Tyr Asn Asp Thr Ser Leu Val Glu
 25 30
 20
 AAT CAT CTC TGC CCT GCC ACA GAG GGG CCC CTC ATG GCC 509
 Asn His Leu Cys Pro Ala Thr Glu Gly Pro Leu Met Ala
 35 40 45
 25
 TCC TTC AAG GCC GTG TTC GTG CCC GTG GCC TAC AGC CTC 548
 Ser Phe Lys Ala Val Phe Val Pro Val Ala Tyr Ser Leu
 50 55 60
 30
 ATC TTC CTC CTG GGC GTG ATC GGC AAC GTC CTG GTG CTG 587
 Ile Phe Leu Leu Gly Val Ile Gly Asn Val Leu Val Leu
 65 70
 35
 GTG ATC CTG GAG CGG CAC CGG CAG ACA CGC AGT TCC ACG 626
 Val Ile Leu Glu Arg His Arg Gln Thr Arg Ser Ser Thr
 75 80 85
 40
 GAG ACC TTC CTG TTC CAC CTG GCC GTG GCC GAC CTC CTG 665
 Glu Thr Phe Leu Phe His Leu Ala Val Ala Asp Leu Leu
 90 95
 45
 CTG GTC TTC ATC TTG CCC TTT GCC GTG GCC GAG GGC TCT 704
 Leu Val Phe Ile Leu Pro Phe Ala Val Ala Glu Gly Ser
 100 105 110
 50
 GTG GGC TGG GTC CTG GGG ACC TTC CTC TGC AAA ACT GTG 743
 Val Gly Trp Val Leu Gly Thr Phe Leu Cys Lys Thr Val
 115 120 125
 55
 ATT GCC CTG CAC AAA GTC AAC TTC TAC TGC AGC AGC CTG 782
 Ile Ala Leu His Lys Val Asn Phe Tyr Cys Ser Ser Leu
 130 135
 60
 CTC CTG GCC TGC ATC GCC GTG GAC CGC TAC CTG GCC ATT 821
 Leu Leu Ala Cys Ile Ala Val Asp Arg Tyr Leu Ala Ile
 140 145 150
 65
 GTC CAC GCC GTC CAT GCC TAC CGC CAC CGC CGC CTC CTC 860
 Val His Ala Val His Ala Tyr Arg His Arg Arg Leu Leu
 155 160
 70
 TCC ATC CAC ATC ACC TGT GGG ACC ATC TGG CTG GTG GGC 899
 Ser Ile His Ile Thr Cys Gly Thr Ile Trp Leu Val Gly
 165 170 175
 75
 TTC CTC CTT GCC TTG CCA GAG ATT CTC TTC GCC AAA GTC 938
 Phe Leu Leu Ala Leu Pro Glu Ile Leu Phe Ala Lys Val
 180 185 190
 80
 AGC CAA GGC CAT CAC AAC AAC TCC CTG CCA CGT TGC ACC 977
 Ser Gln Gly His His Asn Asn Ser Leu Pro Arg Cys Thr

-46-

		195	200	
5	TTC	TCC CAA GAG AAC CAA GCA GAA ACG CAT GCC TGG TTC	1016	
	Phe	Ser Gln Glu Asn Gln Ala Glu Thr His Ala Trp Phe	215	
		205	210	
10	ACC	TCC CGA TTC CTC TAC CAT GTG GCG GGA TTC CTG CTG	1055	
	Thr	Ser Arg Phe Leu Tyr His Val Ala Gly Phe Leu Leu	225	
		220	225	
15	CCC	ATG CTG GTG ATG GGC TGG TGC TAC GTG GGG GTA GTG	1094	
	Pro	Met Leu Val Met Gly Trp Cys Tyr Val Gly Val Val	240	
		230	235	
20	CAC	AGG TTG CGC CAG GCC CAG CGG CGC CCT CAG CGG CAG	1133	
	His	Arg Leu Arg Gln Ala Gln Arg Arg Pro Gln Arg Gln	255	
		245	250	
25	AAG	GCA GTC AGG GTG GCC ATC CTG GTG ACA AGC ATC TTC	1172	
	Lys	Ala Val Arg Val Ala Ile Leu Val Thr Ser Ile Phe	265	
		260	265	
30	TTC	CTC TGC TGG TCA CCC TAC CAC ATC GTC ATC TTC CTG	1211	
	Phe	Leu Cys Trp Ser Pro Tyr His Ile Val Ile Phe Leu	280	
		270	275	
35	GAC	ACC CTG GCG AGG CTG AAG GCC GTG GAC AAT ACC TGC	1250	
	Asp	Thr Leu Ala Arg Leu Lys Ala Val Asp Asn Thr Cys	290	
		285	290	
40	AAG	CTG AAT GGC TCT CTC CCC GTG GCC ATC ACC ATG TGT	1289	
	Lys	Leu Asn Gly Ser Leu Pro Val Ala Ile Thr Met Cys	305	
		295	300	
45	GAG	TTC CTG GGC CTG GCC CAC TGC TGC CTC AAC CCC ATG	1328	
	Glu	Phe Leu Gly Leu Ala His Cys Cys Leu Asn Pro Met	320	
		310	315	
50	CTC	TAC ACT TTC GCC GGC GTG AAG TTC CGC AGT GAC CTG	1367	
	Leu	Tyr Thr Phe Ala Gly Val Lys Phe Arg Ser Asp Leu	330	
		325	330	
55	TCG	CGG CTC CTG ACG AAG CTG GGC TGT ACC GGC CCT GCC	1406	
	Ser	Arg Leu Leu Thr Lys Leu Gly Cys Thr Gly Pro Ala	345	
		335	340	
60	TCC	CTG TGC CAG CTC TTC CCT AGC TGG CGC AGG AGC AGT	1445	
	Ser	Leu Cys Gln Leu Phe Pro Ser Trp Arg Arg Ser Ser	355	
		350	355	
65	CTC	TCT GAG TCA GAG AAT GCC ACC TCT CTC ACC ACG TTC TA	1486	
	Leu	Ser Glu Ser Glu Asn Ala Thr Ser Leu Thr Thr Phe	372	
		360	365	
70	GGTC	CCAGTGTCCC CTTTTATTGC TGCTTTTCCT TGGGGCAGGC	1530	
75	AGTGATGCTG	GATGCTCCTT CCAACAGGAG CTGGGATCCT AAGGGCTCAC	1580	
80	CGTGGCTAAG	AGTGTCTAG GAGTATCCTC ATTTGGGGTA GCTAGAGGAA	1630	
85	CCAACCCCCA	TTTCTAGAAC ATCCCGCGGC CGCCAGCACA CTGGAATTC	1679	

WE CLAIM:

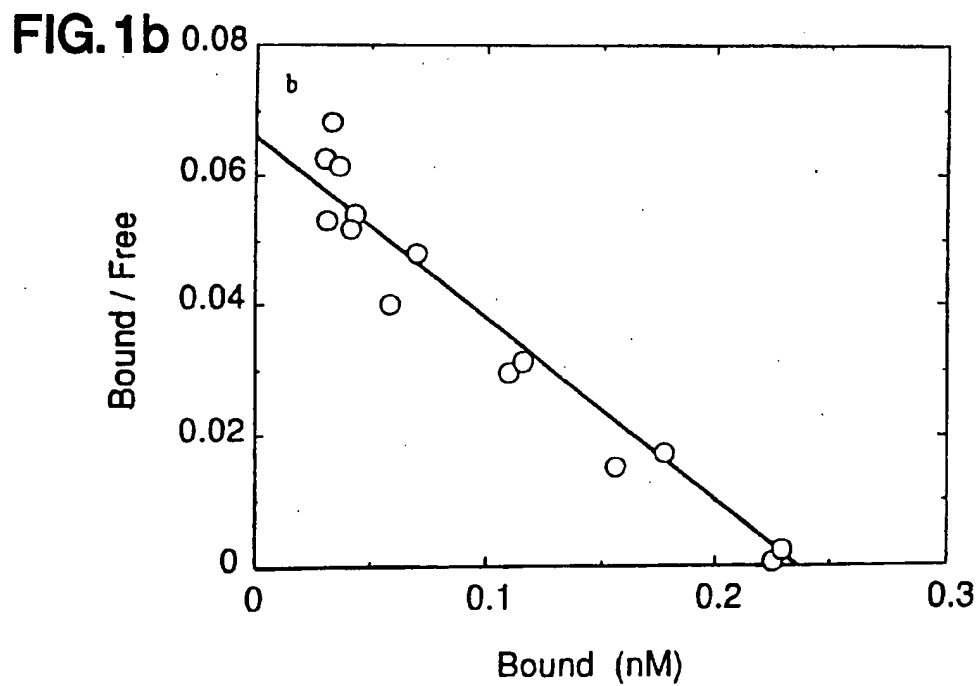
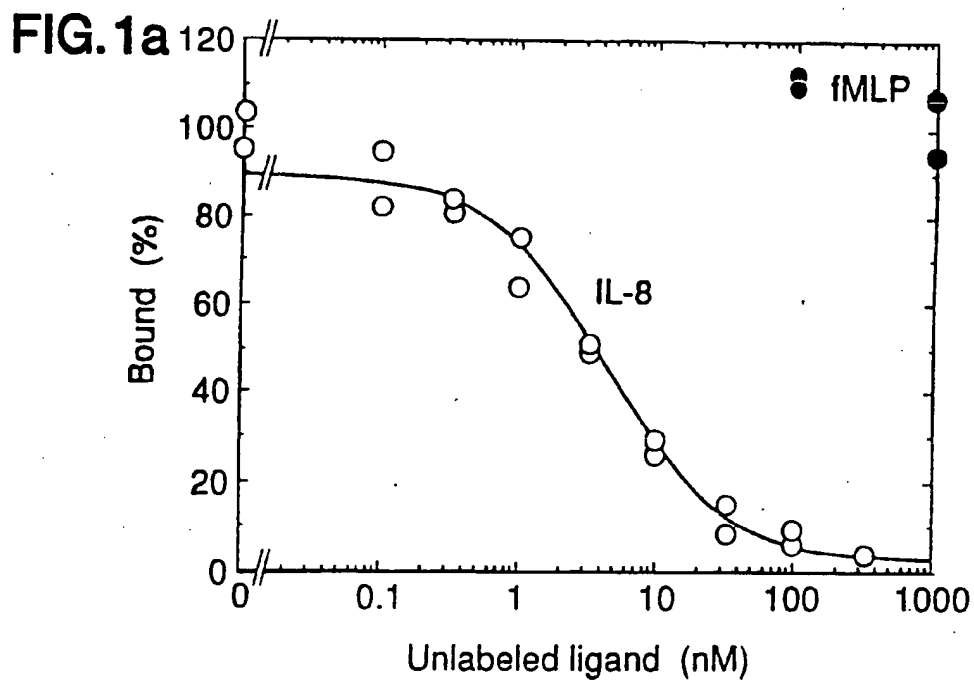
1. An isolated PF4AR polypeptide.
2. A PF4AR polypeptide of claim 1 that is human.
3. A composition comprising the PF4AR polypeptide of claim 1, which composition
5 is free of a contaminating polypeptide of the animal species that is the source of the PF4AR polypeptide.
4. An antibody that is capable of binding the PF4AR polypeptide of claim 1 that does not cross-react with a receptor capable of binding another PF4 superfamily member.
5. An antibody that is capable of binding a PF4AR.
- 10 6. An isolated nucleic acid molecule encoding a PF4AR.
7. The nucleic acid molecule of claim 6 which is DNA and has the translated DNA sequence shown in Figures 2, 4 or 5.
8. The nucleic acid molecule of claim 6 which is DNA and contains greater than about 45 bases.
- 15 9. The nucleic acid molecule of claim 6 further comprising a promoter operably linked to the nucleic acid sequence.
10. An expression vector comprising the nucleic acid sequence of claim 6 operably linked to control sequences recognized by a host cell transformed with the vector.
11. A host cell transformed with the vector of claim 10.
- 20 12. A method of using a nucleic acid molecule encoding a PF4AR, comprising expressing the nucleic acid molecule in a cultured host cell transformed with a vector comprising the nucleic acid molecule encoding the PF4AR operably linked to control sequences recognized by the host cell transformed with the vector, and recovering PF4AR from the host cell.
- 25 13. The method of claim 12 wherein the PF4AR is recovered from the host cell membranes.
14. A method of determining the presence of a PF4AR, comprising hybridizing DNA encoding or complementary to the PF4AR to a test sample nucleic acid and determining the presence of PF4AR DNA.
- 30 15. A method of amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase reaction with nucleic acid encoding or complementary to a PF4AR nucleic acid.
16. A composition comprising a PF4AR of claim 1 and a pharmaceutically acceptable carrier.
- 35 17. A composition comprising a PF4AR antibody and a pharmaceutically acceptable carrier.
18. An isolated polypeptide comprising a PF4AR fused to a polypeptide heterologous to the receptor.

-48-

19. A method comprising transforming a host cell with DNA encoding the IL-8 receptor, culturing the host cell to express the receptor on its surface, harvesting the cells, contacting the cells with an IL-8 variant and determining the biological effect of the variant on the receptor.

5 20. The method of claim 19 wherein the IL-8 variant is an amino acid sequence variant of IL-8.

1 / 28



SUBSTITUTE SHEET

2/28

CCTGGCCGGT GCTTCAGTTA GATCAAACCA TTGCTGAAAC TGAAGAGGAC 50

ATG TCA AAT ATT ACA GAT CCA CAG ATG TGG GAT TTT 86
 Met Ser Asn Ile Thr Asp Pro Gln Met Trp Asp Phe
 1 5 10

GAT GAT CTA AAT TTC ACT GGC ATG CCA CCT GCA GAT GAA 125
 Asp Asp Leu Asn Phe Thr Gly Met Pro Pro Ala Asp Glu
 15 20 25

GAT TAC AGC CCC TGT ATG CTA GAA ACT GAG ACA CTC AAC 164
 Asp Tyr Ser Pro Cys Met Leu Glu Thr Glu Thr Leu Asn
 30 35

AAG TAT GTT GTG ATC ATC GCC TAT GCC CTA GTG TTC CTG 203
 Lys Tyr Val Val Ile Ile Ala Tyr Ala Leu Val Phe Leu
 40 45 50

FIG.2A-1

SUBSTITUTE SHEET

3/28

TM1

CTG AGC CTG CTG GGA AAC TCC CTG GTG ATG CTG GTC ATC 242
Leu Ser Leu Leu Gly Asn Ser Leu Val Met Leu Val Ile
 55 60

TTA TAC AGC AGG GTC GGC CGC TCC GTC ACT GAT GTC TAC 281
Leu Tyr Ser Arg Val Gly Arg Ser Val Thr Asp Val Tyr
 65 70 75

TM2

CTG CTG AAC CTG GCC TTG GCC GAC CTA CTC TTT GCC CTG 320
Leu Leu Asn Leu Ala Leu Ala Asp Leu Leu Phe Ala Leu
 80 85 90

ACC TTG CCC ATC TGG GCC GCC TCC AAG GTG AAT GGC TGG 359
Thr Leu Pro Ile Trp Ala Ala Ser Lys Val Asn Gly Trp
 95 100

SUBSTITUTE SHEET

FIG.2A-2

4/28

ATT TTT GGC ACA TTC CTG TGC AAG GTG GTC TCA CTC CTG 398
 Ile Phe Gly Thr Phe Leu Cys Lys Val Val Ser Leu Leu
 105 110 115

AAG GAA GTC AAC TTC TAC AGT GGC ATC CTG CTG TTG GCC 437
 Lys Glu Val Asn Phe Tyr Ser Gly Ile Leu Leu Ala Ala
 120 125

TGC ATC AGT GTG GAC CGT TAC CTG GCC ATT GTC CAT GCC 476
 Cys Ile Ser Val Asp Arg Tyr Leu Ala Ile Val His Ala
 130 135 140

ACA CGC ACA CTG ACC CAG AAG CGT CAC TTG GTC AAG TTT 515
 Thr Arg Thr Leu Thr Gln Lys Arg His Leu Val Lys Phe
 145 150 155

GTT TGT CTT GGC TGC TGG GGA CTG TCT ATG AAT CTG TCC 554
 Val Cys Leu Gly Cys Trp Gly Leu Ser Met Asn Leu Ser
 160 165

TM3
 TM4

FIG.2A-3

SUBSTITUTE SHEET

5/28

CTG CCC TTC TTC CTT TTC CGC CAG GCT TAC CAT CCA AAC 593
Leu Pro Phe Phe Leu Phe 175
 170 180

AAT TCC AGT CCA GTT TGC TAT GAG GTC CTG GGA AAT GAC 632
 Asn Ser Ser Pro Val Cys Tyr Glu Val Leu Gly Asn Asp
 185 190

ACA GCA AAA TGG CGG ATG GTG TTG CGG ATC CTG CCT CAC 671
 Thr Ala Lys Trp Arg Met Val Leu Arg Ile Leu Pro His
 195 200 205

ACC TTT GGC TTC ATC GTG CCG CTG TTT GTC ATG CTG TTC 710
Thr Phe Gly Phe Ile Val Pro Leu Phe Val Met Leu Phe
 210 215 220

TM5

FIG.2B-1

SUBSTITUTE SHEET

6 / 28

TGC TAT GGA TTC ACC CTG CGT ACA CTG TTT AAG GCC CAC 749
Cys Tyr Gly Phe Thr Leu Arg Thr Leu Phe 230
 225

ATG GGG CAG AAG CAC CGA GCC ATG AGG GTC ATC TTT GCT 788
 Met Gly Gln Lys His Arg Ala Met Arg Val Ile Phe Ala
 235 240 245

TM6
 GTC GTC CTC ATC TTC CTG CTT TGC TGG CTG CCC TAC AAC 827
Val Val Leu Ile Phe Leu Leu Cys Trp Leu Pro Tyr Asn
 250 255

CTG GTC CTG CTG GCA GAC ACC CTC ATG AGG ACC CAG GTG 866
Leu Val Leu Leu Ala Asp Thr Leu Met Arg Thr Gln Val
 260 265 270

ATC CAG GAG ACC TGT GAG CGC CGC AAC ATC GGC CGG 905
 Ile Gln Glu Thr Cys Glu Arg Arg Asn Asn Ile Gly Arg
 275 280 285

FIG.2B-2

SUBSTITUTE SHEET

7/28

GCC CTG GAT GCC ACT GAG ATT CTG GGA TTT CTC CAT AGC 944
 Ala Leu Asp Ala Thr Glu Ile Leu Gly Phe Leu His Ser
 290 295

TM7

TGC CTC AAC CCC ATC ATC TAC GCC TTC ATC GGC CAA AAT 983
 Cys Leu Asn Pro Ile Ile Tyr Ala Phe Ile Gly Gln Asn
 300 305 310

TTT CGC CAT GGA TTC CTC AAG ATC CTG GCT ATG CAT GGC 1022
 Phe Arg His Gly Phe Leu Lys Ile Leu Ala Met His Gly
 315 320

CTG GTC AGC AAG GAG TTC TTG GCA CGT CAT CGT GTT ACC 1061
 Leu Val Ser Lys Glu Phe Leu Ala Arg His Arg Val Thr
 325 330 335

TCC TAC ACT TCT TCG TCT GTC AAT GTC TCT TCC AAC CTC 1100
 Ser Tyr Thr Ser Ser Ser Val Asn Val Ser Ser Asn Leu
 340 345 350

FIG.2B-3

SUBSTITUTE SHEET

8 / 28

TGAAACCAT CGATGAAGGA ATATCTCTTC TCAGAAGGAA AGAATAACCA 1150
ACACCTGAG GTTGTGTGTG GAAGTGATC TGGCTCTGGA CAGGCACTAT 1200
CTGGGTTTG GGGGGACGCT ATAGGATGTG GGGAAGTTAG GAACTGGTGT 1250
CTTCAGGGC CACACCAACC TTCTGAGGAG CTGTTGAGGT ACCTCCAAGG 1300
ACCGGCCTTT GCACCTCCAT GGAACGAAG CACCATCATT CCCGTTGAAC 1350
GTCACATCTT TAACCCACTA ACTGGCTAAT TAGCATGGCC ACATCTGAGC 1400
CCCGAATCTG ACATTAGATG AGAGAACAGG GCTGAAGCTG TGTCCTCATG 1450

FIG.2C-I

SUBSTITUTE SHEET

9/28

AGGGCTGGAT GCTCTCGTTG ACCCTCACAG GAGCATCTCC TCAACTCTGA 1500
GTGTTAAGCG TTGAGCCACC AAGCTGGTGG CTCTGTGTGC TCTGATCCGA 1550
GCTCAGGGGG GTGGTTTTC CATCTCAGGT GTGTTGCAGT GTCTGCTGGA 1600
GACATTGAGG CAGGCACTGC CAAACATCA ACCTGCCAGC TGGCCTGTG 1650
AGGAGCTGGA AACACATGTT CCCCTTGGG GTGGTGGATG AACAAAGAGA 1700
AAGAGGGTTT GGAAGCCAGA TCTATGCCAC AAGAACCCC TTTACCCCA 1750

FIG.2C-2

SUBSTITUTE SHEET

10/28

TGACCAACAT CGCAGACACA TGTGCTGGCC ACCTGCTGAG CCCCAGTGG 1800

AACGAGACAA GCAGCCCTTA GCCCTTCCCC TCTGCAGCTT CCAGGCTGGC 1850

GTGCAGCATC AGCATCCCTA GAAAGCCATG TGCAGCCACC AGTCCATTGG 1900

GCAGGCAGAT GTTCCTAATA AAGCTTCTGT TCC 1933

FIG.2C-3

SUBSTITUTE SHEET

11/28

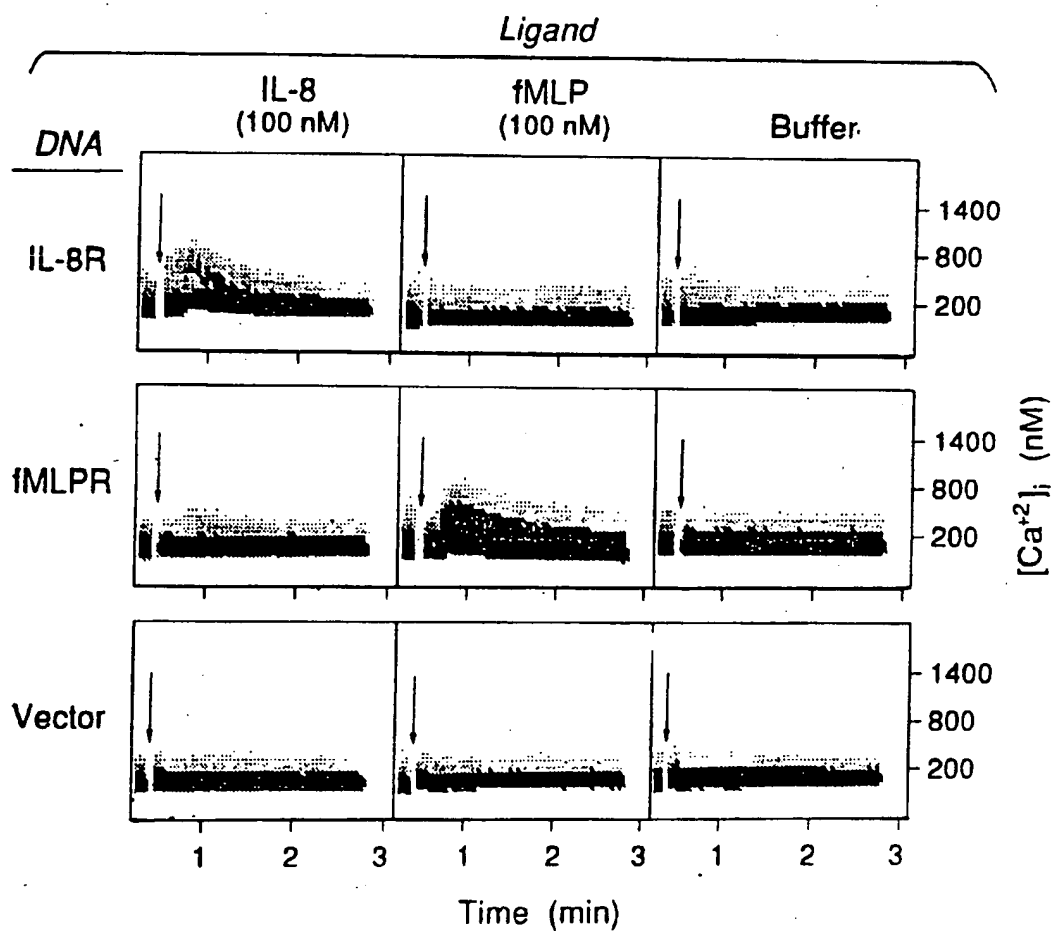


FIG.3a

SUBSTITUTE SHEET

12/28

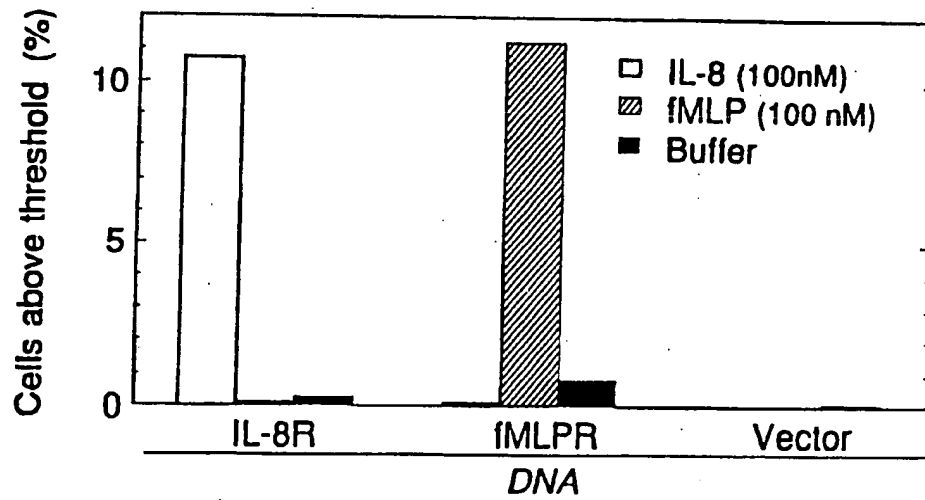


FIG.3b

SUBSTITUTE SHEET

13/28

GAATTCCAGT GTGCTGGCGG CGCGGCGCAA AGTGACGCCG AGGGCCTGAG 50

TGCTCCAGTA GCCACCGCAT CTGGAGAACC AGCGGTTACC ATG GAG 96

Met Glu

1

GGG ATC AGT ATA TAC ACT TCA GAT AAC TAC ACC GAG GAA 135

Gly Ile Ser Ile Tyr Thr Ser Asp Asn Tyr Thr Glu Glu 15

10

ATG GGC TCA GGG GAC TAT GAC TCC ATG AAG GAA CCC TGT 174

Met Gly Ser Gly Asp Tyr Asp Ser Met Lys Glu Pro Cys 25

20

TTC CGT GAA GAA AAT GCT AAT TTC AAT AAA ATC TTC CTG 213

Phe Arg Glu Glu Asn Ala Asn Phe Asn Lys Ile Phe Leu 40

35

CCC ACC ATC TAC TCC ATC ATC TTC TTA ACT GGC ATT GTG 252

Pro Thr Ile Tyr Ser Ile Ile Phe Leu Thr Gly Ile Val 50

45

FIG.4A-1

SUBSTITUTE SHEET

14/28

GCC AAT GGA TTG GTC ATC CTG GTC ATG GGT TAC CAG AAG	291
Gly Asn Gly Leu Val Ile Leu Val Met Gly Tyr Gln Lys	65
55	
AAA CTG AGA AGC ATG ATG ACG GAC AAG TAC AGG CTG CAC CTG	330
Lys Leu Arg Ser Met Thr Asp Lys Tyr Arg Leu His Leu	80
70	
TCA GTG GCC GAC CTC CTC TTT GTC ATC ACG CTT CCC TTC	369
Ser Val Ala Asp Leu Leu Phe Val Ile Thr Leu Pro Phe	90
85	
TGG GCA GTT GAT GCC GTG GCA AAC TGG TAC TTT GGG AAC	408
Trp Ala Val Asp Ala Val Ala Asn Trp Tyr Phe Gly Asn	105
95	
100	

FIG.4A-2

SUBSTITUTE SHEET

15/28

TTC CTA TGC AAG GCA GTC CAT GTC ATC TAC ACA GTC AAC	447
Phe Leu Cys Lys Ala Val His Val Ile Tyr Thr Val Asn	
	110
	115
CTC TAC AGC AGT GTC CTC ATC CTG GCC TTC ATC AGT CTG	486
Leu Tyr Ser Ser Val Leu Ile Leu Ala Phe Ile Ser Leu	
	120
	125
GAC CGC TAC CTG GCC ATC GTC CAC GCC ACC AAC AGT CAG	525
Asp Arg Tyr Leu Ala Ile Val His Ala Thr Asn Ser Gln	
	135
	140
AGG CCA AGG AAG CTG TTG GCT GAA AAG GTG GTC TAT GTT	564
Arg Pro Arg Lys Leu Leu Ala Glu Lys Val Val Tyr Val	
	150
	155
GGC GTC TGG ATC CCT GCC CTC CTG CTG ACT ATT CCC GAC	603
Gly Val Trp Ile Pro Ala Leu Leu Thr Ile Pro Asp	
	160
	165
	170

FIG.4A-3

SUBSTITUTE SHEET

16/28

TTC ATC TTT GCC AAC GTC AGT GAG GCA GAT GAC AGA TAT	642
Phe Ile Phe Ala Asn Val Ser Glu Ala Asp Arg Tyr	
	175
	180
ATC TGT GAC CGC TTC TAC TCC CCC AAT GAC TTG TGG GTG GTT	681
Ile Cys Asp Arg Phe Tyr Pro Asn Asp Leu Trp Val Val	
	185
	190
	195
GTG TTC CAG TTT CAG CAC ATC ATG GTT GGC CTT ATC CTG	720
Val Phe Gln Phe Gln His Ile Met Val Gly Leu Ile Leu	
	200
	205
	210
CCT GGT ATT GTC ATC CTG TCC TGC TAT TGC ATT ATC ATC	759
Pro Gly Ile Val Ile Ile Leu Ser Cys Tyr Cys Ile Ile Ile	
	215
	220
TCC AAG CTG TCA CAC CAC TCC AAG GGC CAC CAG AAG CGC AAG	798
Ser Lys Leu Ser His His Ser Lys Gly His Gln Lys Arg Lys	
	225
	230
	235

FIG.4B-I

SUBSTITUTE SHEET

17/28

```

GCC CTC AAG ACC ACA GTC ATC CTC ATC CTG GCT TTC TTC 837
Ala Leu Lys Thr Thr Val Ile Ile Leu Ala Phe Phe
240 245

GCC TGT TGG CTG CCT TAC TAC ATT GGG ATC AGC ATC GAC 876
Ala Cys Trp Leu Pro Tyr Tyr Ile Gly Ile Ser Ile Asp
250 255

TCC TTC ATC CTC CTG GAA ATC ATC AAG CAA GGG TGT GAG 915
Ser Phe Ile Leu Leu Glu Ile Ile Lys Gln Gly Cys Glu
265 270

TTT GAG AAC ACT GTG CAC AAG AAG TGG ATT TCC ATC ACC GAG 954
Phe Glu Asn Thr Val His Lys Lys Trp Ile Ser Ile Thr Glu
280 285

GCC CTA GCT TTC TTC CAC TGT TGT CTG AAC CCC ATC CTC 993
Ala Leu Ala Phe Phe His Cys Cys Leu Asn Pro Ile Leu
290 300

```

FIG.4B-2

SUBSTITUTE SHEET

18/28

TAT GCT TTC CTT GGA GCC AAA TTT AAA ACC TCT GCC CAG 1032
 Tyr Ala Phe Leu Gly Ala Lys Phe Lys Thr Ser Ala Gln
 305 310

CAC GCA CTC ACC TCT GTG AGC AGA GGG TCC AGC CTC AAG 1071
 His Ala Leu Thr Ser Val Ser Arg Gly Ser Ser Leu Lys
 315 320 325

ATC CTC TCC AAA GGA AAG CGA GGT GGA CAT TCA TCT GTT 1110
 Ile Leu Ser Lys Gly Lys Arg Gly Gly His Ser Ser Val
 330 335 340

TCC ACT GAG TCT GAG TCT TCA AGT TTT CAC TCC AGC TAAC 1150
 Ser Thr Glu Ser Glu Ser Ser Ser Phe His Ser Ser
 345 350 352

ACAGATGTAA AAGACTTTT TTTATACGAT AAATAACTTT TTTTAAGTT 1200

FIG.4B-3

SUBSTITUTE SHEET

19/28

ACACATTTT CAGATATAA A GACTGACCA ATATTGTACA GTTTTATTG 1250
CTTGTTGGAT TTTTGTCTTG TGTTCCTTA GTTTTGTGA AGTTAATTG 1300
ACTTATTAT ATAAATTTT TTTGTTTCAT ATTGATGTGT GTCTAGGCAG 1350
GACCTGTGGC CAAGTCTTA GTTGCTGTAT GTCTCGTGGT AGGACTGTAG 1400
AAAAGGGAAC TGAACATTCC AGAGCGTGA GTGAATCAG TAAAGCTAGA 1450
AATGATCCCC AGCTGTTTAT GCATAGATAA TCTCTCCATT CCCGTGAAC 1500

FIG.4C-I

SUBSTITUTE SHEET

20/28

GTTTTTCCTG TTCTTAAGAC GTGATTTTGC TGTAGAAGAT GGCACTTATA 1550

ACCAAAGCCC AAAGTGGTAT AGAAATGCTG GTTTTTCAGT TTTCAGGAGT 1600

GGGTTGATT CAGCACCTAC AGTGACAGT CTTGTATTAA GTTGTATA 1650

AAAGTACATG TTAAACTTAA AAAAAAAAAA AAAAAAAAAA 1700

AAAAAAAAAA AAAGCGCCG CCAGCACACT GGAATTC 1737

FIG.4C-2

SUBSTITUTE SHEET

21/28

GAATCCAGT GTGCTGGCGG CCGCCAGTG TGCTGGCGG GGCAGTTGAG 50
GGAAGGACA GAGGTATGA GTGCCTGCAA GAGTGGCAGC CTGGAGTAGA 100
GAAACACTA AAGGTGGAGT CAAAGACCT GAGTCAAGT CCCAGCTCTG 150
CCACTGGTA GCTGTGGGAT CTCGGAAAAG ACCCAGTGAA AAAAAAAA 200
AAAGTGATGA GTGTGAGGC AGGTCGCGC CCTACTGCCT CAGGAGACGA 250
TGGCAGCTC ATTTGCTTA ATTTGCAGCT GACGGCTGCC ACCTCTCTAG 300

FIG.5A-I

SUBSTITUTE SHEET

22/28

AGGCACCTGG CGGGGAGCCT CTCAACATAA GACAGTGACC AGTCTGGTGA 350

CTCACAGCCG GCACAGCC ATG AAC TAC CCG CTA ACG CTG GAA 392
Met Asn Tyr Pro Leu Thr Leu Glu
1 5

ATG GAC CTC GAG AAC CTG GAG GAC CTG TTC TGG GAA CTG 431
Met Asp Leu Glu Asn Leu Glu Asp Leu Phe Trp Glu Leu
10 15 20

GAC AGA TTG GAC AAC TAT AAC GAC ACC TCC CTG GTG GAA 470
Asp Arg Leu Asp Asn Tyr Asn Asp Thr Ser Leu Val Glu
25 30

AAT CAT CTC TGC CCT GCC ACA GAG GGG CCC CTC ATG GCC 509
Asn His Leu Cys Pro Ala Thr Glu Gly Pro Leu Met Ala
35 40 45

FIG.5A-2

SUBSTITUTE SHEET

23/28

TCC	TTC	AAG	GCC	GTG	TTC	GTG	CCC	GTG	GCC	TAC	AGC	CTC	548
Ser	Phe	Lys	Ala	Val	Phe	Val	Pro	Val	Ala	Tyr	Ser	Leu	
		50					55					60	
ATC	TTC	CTC	CTG	GGC	GTG	ATC	GGC	AAC	GTC	CTG	GTG	CTG	587
Ile	Phe	Leu	Leu	Gly	Val	Ile	Gly	Asn	Val	Leu	Val	Leu	
				65					70				
GTG	ATC	CTG	GAG	CGG	CAC	CGG	CAG	ACA	CGC	AGT	TCC	ACG	626
Val	Ile	Leu	Glu	Arg	His	Arg	Gln	Thr	Arg	Ser	Ser	Thr	
		75				80					85		
GAG	ACC	TTC	CTG	TTC	CAC	CTG	GCC	GTG	GCC	GAC	CTC	CTG	665
Glu	Thr	Phe	Leu	Phe	His	Leu	Ala	Val	Ala	Asp	Leu	Leu	
			90					95					
CTG	GTC	TTC	ATC	TTG	CCC	TTT	GCC	GTG	GCC	GAG	GGC	TCT	704
Leu	Val	Phe	Ile	Leu	Pro	Phe	Ala	Val	Ala	Glu	Gly	Ser	
100					105					110			

FIG.5A-3

SUBSTITUTE SHEET

GTG GGC	TGG	GTC	CTG	GGG	ACC	TTC	CTC	TGC	AAA	ACT	GTG	743
Val Gly	Trp	Val	Leu	Gly	Thr	Phe	Leu	Cys	Lys	Thr	Val	
	115					120					125	
ATT GCC	CTG	CAC	AAA	GTC	AAC	TTC	TAC	TGC	AGC	AGC	CTG	782
Ile Ala	Leu	His	Lys	Val	Asn	Phe	Tyr	Cys	Ser	Ser	Leu	
			130					135				
CTC CTG	GCC	TGC	ATC	GCC	GTG	GAC	CGC	TAC	CTG	GCC	ATT	821
Leu Leu	Ala	Cys	Ile	Ala	Val	Asp	Arg	Tyr	Leu	Ala	Ile	
	140				145					150		
GTC CAC	GCC	GTC	CAT	GCC	TAC	CGC	CAC	CGC	CGC	CTC	CTC	860
Val His	Ala	Val	His	Ala	Tyr	Arg	His	Arg	Arg	Leu	Leu	
		155				160						
TCC ATC	CAC	ATC	ACC	TGT	GGG	ACC	ATC	TGG	CTG	GTG	GGC	899
Ser Ile	His	Ile	Thr	Cys	Gly	Thr	Ile	Trp	Leu	Val	Gly	
165				170							175	

FIG. 5B-1

SUBSTITUTE SHEET

25/28

TTC CTC CTT GCC TTG CCA GAG ATT CTC TTC GCC AAA GTC	938
Phe Leu Leu Ala Leu Pro Glu Ile Leu Phe Ala Lys Val	180 185 190
AGC CAA GGC CAT CAC AAC AAC TCC CTG CCA CGT TGC ACC	977
Ser Gln Gly His His Asn Ser Leu Pro Arg Cys Thr	195 200
TTC TCC CAA GAG AAC CAA GCA GAA ACG ACG CAT GCC TGG TTC	1016
Phe Ser Gln Glu Asn Gln Ala Glu Thr His Ala Trp Phe	205 210 215
ACC TCC CGA TTC CTC TAC CAT GTG GCG GGA TTC CTG CTG	1055
Thr Ser Arg Phe Leu Tyr His Val Ala Gly Phe Leu Leu	220 225
CCC ATG CTG GTG ATG GGC TGG TGC TAC GTG GGG GTA GTG	1094
Pro Met Leu Val Met Gly Trp Cys Tyr Val Gly Val Val	230 235 240

FIG.5B-2

SUBSTITUTE SHEET

26/28

CAC	AGG	TTG	CGC	CAG	GCC	CAG	CGG	CGC	CCT	CAG	CGG	CAG	1133
His	Arg	Leu	Arg	Gln	Ala	Gln	Arg	Arg	Pro	Gln	Arg	Gln	255
		245		250									
AAG	GCA	GTC	AGG	GTG	GCC	ATC	CTG	GTG	ACA	AGC	ATC	TTC	1172
Lys	Ala	Val	Arg	Val	Ala	Ile	Leu	Val	Thr	Ser	Ile	Phe	
		260							265				
TTC	CTC	TGC	TGG	TCA	CCC	TAC	CAC	ATC	GTC	ATC	TTC	CTG	1211
Phe	Leu	Cys	Trp	Ser	Pro	Tyr	His	Ile	Val	Ile	Phe	Leu	
	270					275					280		
GAC	ACC	CTG	GCG	AGG	CTG	AAG	GCC	GTG	GAC	AAT	ACC	TGC	1250
Asp	Thr	Leu	Ala	Arg	Leu	Lys	Ala	Val	Asp	Asn	Thr	Cys	
		285						290					

FIG.5B-3

SUBSTITUTE SHEET

27/28

FIG.5C-1

AAG CTG AAT GGC TCT CTC CCC GTG GCC ATC ACC ATG TGT	1289
Lys Leu Asn Gly Ser Leu Pro Val Ala Ile Thr Met Cys	
295	305
GAG TTC CTG GGC CTG GGC CAC TGC TGC TGC TGC ATG	1328
Glu Phe Leu Gly Leu Ala His Cys Cys Cys Leu Asn Pro Met	
310	320
CTC TAC ACT TTC GCC GGC GTG AAG TTC CGC AGT GAC CTG	1367
Leu Tyr Thr Phe Ala Gly Val Lys Phe Arg Ser Asp Leu	
325	330
TCG CGG CTC CTG ACG AAG CTG GGC TGT ACC GGC CCT GCC	1406
Ser Arg Leu Leu Thr Lys Leu Gly Cys Thr Gly Pro Ala	
335	345
TCC CTG TGC CAG CTC TTC CCT AGC TGG CGC AGG AGC AGT	1445
Ser Leu Cys Gln Leu Phe Pro Ser Trp Arg Ser Ser	
350	355

SUBSTITUTE SHEET

28/28

CTC TCT GAG TCA GAG AAT GCC ACC TCT CTC ACC ACC TTC TA 1486
Leu Ser Glu Ser Glu Asn Ala Thr Ser Leu Thr Phe
360 365 370 372

GGTC CCAGTGTCCC CTTTATTGC TGCTTTTCCT TGGGGCAGGC 1530

AGTGATGCTG GATGCTCCTT CCAACAGGAG CTGGGATCCT AAGGGCTCAC 1580

CGTGGCTAAG AGTGTCCCTAG GAGTATCCTC ATTGGGGTA GCTAGAGGAA 1630

CCAACCCCCA TTTCTAGAAC ATCCCGGGC CGCCAGCACA CTGGAATTC 1679

FIG.5C-2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 92/02317

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07K15/00; C12N5/10;	A61K37/02; C12Q1/68;	C12P21/08; A61K39/395 C12N15/12
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N ; A61K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 33, 25 November 1990, BALTIMORE MD, USA pages 20061 - 20064; K. THOMAS ET AL.: 'Molecular cloning of the fMet-Leu-Phe receptor from neutrophils.' cited in the application see abstract ---	1,3,6, 8-14
X,P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 179, no. 2, 16 September 1991, NEW YORK, USA pages 784 - 789; M. BECKMANN ET AL.: 'Molecular characterization of the interleukin-8 receptor.' see the whole document ---	1,3,6, 8-14
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
17 AUGUST 1992		
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	NOOIJ F.J.M. 